Directed Development of *Bacillus megaterium* for Applications in Recombinant Protein Production

Von der Fakultät für Lebenswissenschaften
der Technischen Universität Carolo-Wilhelmina
zu Braunschweig
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D i s s e r t a t i o n

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Yang, Y., Biedendieck, R., Wang, W., **Gamer, M.**, Malten, M., Jahn, D., Deckwer, W.D. (2006) High yield recombinant penicillin G amidase production and export into the growth medium using *Bacillus megaterium*. *Microb Cell Fact.* 28;5:36.

Malten, M., Biedendieck, R., **Gamer, M.**, Drews, A.C., Stammen, S., Buchholz, K., Dijkhuizen, L., Jahn, D. (2006) A *Bacillus megaterium* plasmid system for the production, export, and one-step purification of affinity-tagged heterologous levansucrase from growth medium. *Appl Environ Microbiol.* 72(2):1677-1679

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Gamer, M., Biedendieck, R., Stammen, S., Fröde, D., Yang, Y., Jahn, D. *Bacillus megaterium* – ein alternatives Expressionssystem (Vortrag). BASF SE, Ludwigshafen, Deutschland, 2007.

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ABBREVIATIONS

bp	base pairs
BCIP	5-Brom-4-chlor-3-indolyl phosphate
dH_2O	deionised water
ddH_2O	double-desilled water
DMF	N,N-dimethylformamide
DNA	deoxyribonucleic acid
D.N.S.	dinitrosalicylic acid
dsDNA	double stranded DNA
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EDTA	ethylenediaminetetraacetic acid
e.g.	*exempli gratia* (for example)
et al.	*et alteri* (and others)
for	forward
g	*centrifugation*: earth gravity
	weight: gram
GFP	green fluorescent protein
h	hour
i.e.	*id est* (that is to say)
kb	kilo base pairs
KBD	keratin-binding domain
LB	Luria Bertani
m	milli
M	molar [mol l^{-1}]
µ	micro
Mbp	mega base pairs
MCS	multiple cloning site
min	minute
MOPS	3-(N-morpholino)-propan sulfonacid
M_r	relative molecular weight
NBT	nitroblue tetrazolium
OD_λ	optical density at wavelength λ in nm
ORF	open reading frame
PBS	phosphate-buffered saline
PCR	polymerase chain reaction

PEG 6000	polyethylen glycol with M_r of 6000
PMF	proton motive force
PVDF	polyvinylidene difluoride
rev	reverse
RNA	ribonucleic acid
RNase	ribonuclease
rpm	rotations per minute
RT	room temperature
s	second
SDS	Na dodecyl sulphate
SDS-PAGE	Na dodecyl sulphate polyacrylamide gel electrophoresis
SP	signal peptide
ssb	single strand DNA binding
TB	terrific broth
TEMED	N,N,N',N',-tetramethylen diamine
TEV	tobacco etch virus
T_m	hybridisation temperature
Tris	tris-(hydroxymethyl)-aminomethane
Tween 20	polyethylene glycol sorbitan monolaurate
T7 RNAP	T7 RNA polymerase
U	units
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume

1 INTRODUCTION

1.1 White Biotechnology

The term “white” or “industrial biotechnology” was coined in 2003 by the European Association for Bioindustries (EuropaBio) to define all industrially bio-based processes that are not covered by red (medicine) or green (plants) biotechnology. White biotechnology has its roots in ancient human history when humans first started to apply microorganisms in the fermentation of food and drinkables. Today, products derived from white biotechnology are ubiquitously present. Many enzymes are used in a wide range of daily applications (*e.g.* as additives in detergents or in food processing). Enzymes have the advantage of being highly selective biocatalysts which can be used in minimal quantities to catalyse chemical reactions at ambient temperatures and reaction conditions. Due to their own chiral nature they are increasingly the alternative of choice for the regio- and stereoselective synthesis of fine chemicals in the pharamceutical and chemical industry.

In 2003, the global industrial enzyme sales were estimated at 2.3 billion dollars with main profits in the areas of detergents, food, feed and textiles. Vitamins, polymers and non-catalytic performance proteins are additional products of modern white biotechnology with strongly increasing economic importance. Representatives of the world’s leading chemical companies (among them the German companies BASF SE, Ludwigshafen; Evonik Industries AG, Essen; Henkel AG, Düsseldorf), analysts as well as policy-makers are convinced that the application of white biotechnology has the potential to impact industrial production processes on a global scale. According to a detailed market analysis carried out by McKinsey & Company, white biotechnology will contribute to 10 - 20% of the entire chemical market in 2010 with annual growth rates of 11 - 22 billion Euro (Frazzetto 2003). Especially, in times of scarcity of resources and escalating prices for petroleum on the world market, smart and innovative technologies are needed to reduce environmental pollution and resource consumption by building up sustainable biomass-based value chains. Therefore, the long-term goal of white biotechnology is the competitive generation of energy out of renewable resources, replacement of conventional production pipelines by bio-based processes and the creation of novel high-value bioproducts with unique characteristics.

1.2 Eukaryotic Proteins of Industrial Importance

Recombinant proteins of eukaryotic origin are of increasing economic importance. Most importantly, the pharmaceutical industry seeks to apply human proteins as therapeutics in clinical applications. Since the first launch of recombinant human insulin (Humulin®, Eli Lilly and Company, Indianapolis, USA) and recombinant human growth hormone (Protropin®, Genentech, San Francisco, USA) in the early 1980's, diverse new therapeutic proteins have entered the pharmaceutical market. Among those are recombinant antibodies which represent the fastest growing class of therapeutic proteins and which are used for the therapy of multiple diseases (Duebel 2007). Biopharmaceuticals are high-value products which are produced in most cases by recombinant cell-lines in elaborate time- and cost intensive processes. However, besides their application in classical red biotechnology, the unique features of catalytic and non-catalytic eukaryotic proteins are increasingly in the focus of white biotechnology. Here, the recombinant production of eukaryotic proteins in industrial scale is highly sensitive towards production expenses making prokaryotic hosts the alternative of choice for the high-level production of desired proteins.

The BASF SE (Ludwigshafen, Germany) represents one of the world's biggest chemical companies and is actively engaged in the application of white biotechnology for the discovery of novel biomaterials. In particular, non-catalytic proteins are in the focus of main research. Two classes of proteins originated from eukaryotic organisms are thought to have the potential for a broad industrial application - human keratin binding domains and fungal hydrophobins. Those so-called performance proteins are designed to be applied as additives in cosmetics and for applications in material sciences.

1.2.1 Human Keratin-Binding-Domains

Research on chemically modified human keratin-binding domains (KBD) as additives in cosmetics started at the BASF SE in 2004 (personal communication with Dr. Heiko Barg, BASF SE, Germany). The innovative idea behind this project was to recombinantly produce proteins with high selective affinities towards human keratins in a suitable microbial host. By adding an additional linker peptide with a chemically reactive cysteine residue to the KBD, chemical compounds like dyes or pharmaceutically active substances (*e.g.* panthenol) should be later on covalently coupled to the recombinant protein (Figure 1.1). Since the product should be applied as additive in cosmetics or pharmaceuticals for hair and skin, a KBD of human origin was

chosen as target. Based on literature search, a protein named desmoplakin was identified as an interesting candidate (Fontao *et al.* 2003).

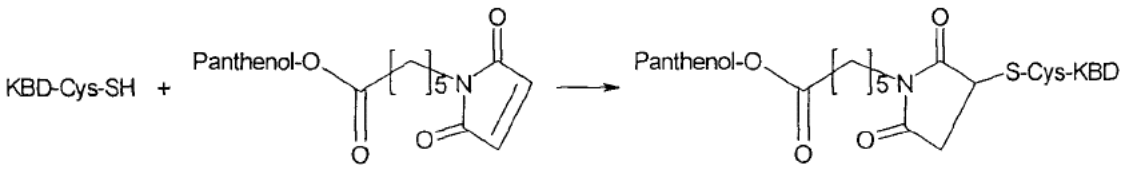

Figure 1.1: Coupling reaction of activated panthenol onto a cysteine residue of the KBD (Barg *et al.* 2007a).

Desmoplakin is the main constituent of the so-called desmosomes in human ephithelial cells. These supramolecular complexes are cell junctions that are predominantly found in the epidermis and heart where they play a key role in maintaining the structural integrity and contribute to cellular strength (Green and Simpson 2007). Desmoplakin itself is a large protein consisting of 2871 amino acids. Its function within the desmosomes is to connect the intermediate filaments with this supramolecular complex. Analysis on the molecular level revealed that desmoplakin can be devided into three parts each mediating a distinct functional property (Stappenbeck *et al.* 1993):

- The N-terminal domain connects desmoplakin with proteins of the desmosomal complex (mainly plakoglobin and plakophilin).
- The central rod domain is responsible for the oligomerisation of individual desmoplakin proteins into homodimers.
- The C-terminal domain mediates binding to the intermediate filaments, such as epidermal keratins, vimentin and desmin (Fontao *et al.* 2003).

The C-terminal domain can be further divided into three subdomains denoted A, B and C (Figure 1.2). Each of these subdomains consists of 4.5 copies of a 38 amino acid repeat motif and is interrupted by intervening sequences of varying length. Crystallographic studies showed that the B and C subdomains form a globular structure with a unique fold (Choi *et al.* 2002).

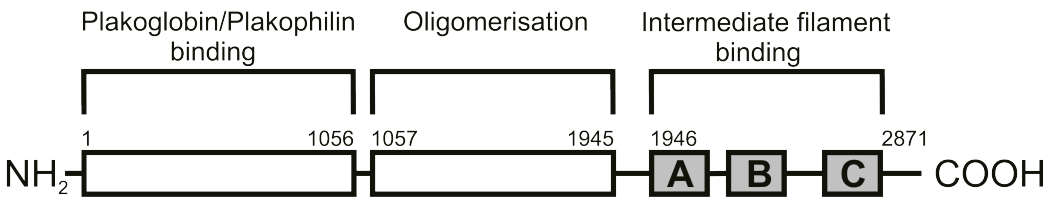

Figure 1.2: Domain structure of desmoplakin (modified according to Choi *et al.*, 2002). The primary structure of desmoplakin can be divided into three major regions each mediating a distinct function. Numbers denote amino acid residues.

1.2.2 Fungal Hydrophobins

Another class of proteins lying in the research focus of the BASF SE are hydrophobins (Subkowski *et al.* 2006). Hydrophobins are highly surface active proteins and are exclusively produced by filamentous fungi where they cover cell-walls, spores and fruiting bodies (Figure 1.3). The biological function of hydrophobins in the live cycle of filamentous fungi is thought to be diverse. By lowering the surface tension of water, they are thought to play a role in the formation of aerial hyphae and fruiting bodies (Wosten and Willey 2000). Moreover, they are involved in the attachment of hyphae to hydrophobic surfaces during symbiotic or pathogenic interactions (Ebbole 1997; Tagu *et al.* 1996).

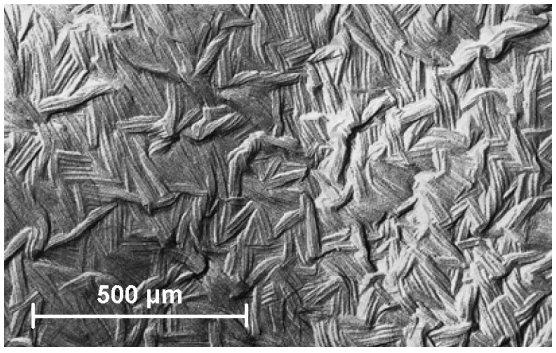

Figure 1.3: Transition electron microscopic image of *Noraspora crassa* spore surface (Dempsey and Beever 1979). Hydrophobins self-assemble at the cell wall-air interface into rodlets.

The first hydrophobin genes were discovered almost two decades ago. Based on their primary protein sequences, these small fungal proteins with a relative molecular size of about 10,000 were named hydrophobins (Schuren and Wessels 1990; Wessels *et al.* 1991). According to differences in their physical properties, they were grouped into two classes. Class I hydrophobins form highly insoluble polymers whereas class II hydrophobins form polymers that are soluble in some organic solvents. Besides their high content of hydrophobic amino acids, their main unifying feature is the presence of eight cysteine residues. Structural analysis showed that the surface of hydrophobins exists of a hydrophobic patch consisting of hydrophobic aliphatic amino acid residues and a hydrophilic part both contributing to their amphiphilic nature (Figure 1.4) (Sunde *et al.* 2008). The presentation of hydrophobic residues on the surface of a protein is a rather uncommon phenomenon since hydrophobic amino acids in soluble proteins typically form hydrophobic cores that stabilise the protein structures. To compensate for the destabilising effect of the hydrophobic patch, the structure of hydrophobins is strongly stabilised by disulfide bridges.

Due to their amphiphilic nature as well as the unique and remarkable property of hydrophobins to self-assemble into rodlets and fibrils, diverse application possibilities were suggested and demonstrated. Inventions range from the use as surface coatings, as anti-fouling agents or as emulsifiers in food processing (Hektor and Scholtmeijer 2005; Montag *et al.* 2006; Keenan *et al.* 2006).

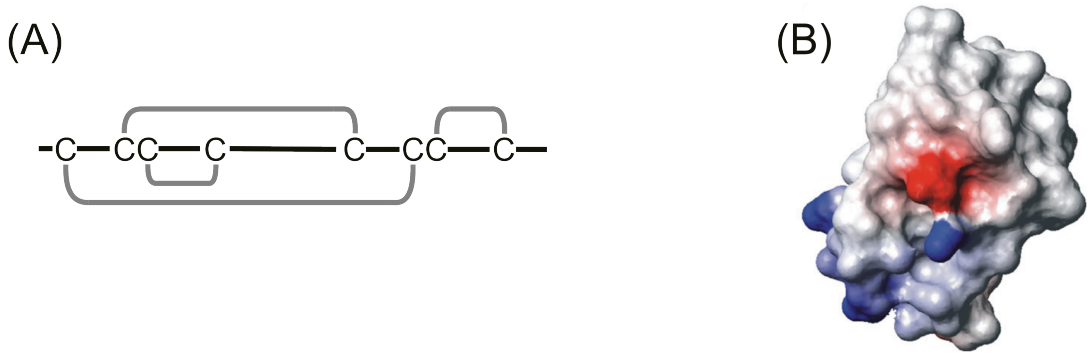

Figure 1.4: (A) Pattern of disulfide bridges in hydrophobins. All eight cysteine residues are involved in the formation of a characteristic pattern of disulfide bridges which highly stabilises the structure. **(B) Electrostatic surface structure of the hydrophobin HFBI from *Trichoderma reesei* (Sunde *et al.* 2008).** Uncharged residues are shown in grey, negatively and positively charged residues are illustrated in red and blue, respectively. The hydrophobic patch is located on the "top" of the molecule.

1.3 Microorganisms for Recombinant Protein Production

Microorganisms, such as bacteria, yeast or multicellular fungi, are often used for the production of recombinant proteins in white biotechnology. These organisms have many advantages compared to more complex eukaryotic systems like cell lines or plants. Former mentioned grow on a variety of cheap substrates, have short generation times and are often easy to genetically be manipulated.

Research on the gastrointestinal bacterium *Escherichia coli* has been done for decades, making it the best studied prokaryotic organism. Molecular tools such as artificial transformation strategies, high-copy number plasmids and gene-knockout systems have been developed allowing for an efficient recombinant protein production. The heterologous production of human insulin with *E. coli* in industrial scale is probably the most impressive example for the use of bacteria as "molecular factories". However, in some cases the Gram-negative nature of *E. coli* limits its use as recombinant protein production host. The outer membrane impedes an efficient release of target proteins into the culture supernatant. Therefore, recombinant proteins are targeted in *E. coli* either intracellularly or to the periplasmic space. Depending on a protein's nature, its intracellular localisation can lead to misfolding and the formation of insoluble protein aggregates. Especially, the correct formation of disulfide bonds is hindered in the

reductive cytosolic milieu. Moreover, in order to purify a given protein to homogeneity, the host cells have to be disrupted and the recombinant protein has to be separated from the cell debris and all cellular components. This whole process, in industrial biotechnology termed downstream processing, is a complex time consuming task which significantly contributes to production costs.

Gram-positive bacteria lack an outer membrane and are known for their potential to target proteins to the extracellular space. Their extracellular localisation can prevent problems associated with folding and allows for a reduction in time and expenses of downstream processing. Especially, species of the genus *Bacillus* are applied in white biotechnology (Schallmey *et al.* 2004).

For more than 50 years, applied and fundamental research has been carried out using *Bacillus subtilis*. Due to its ability to develop genetic competence for DNA binding and uptake, it is genetically highly amenable making it to the model organism of Gram-positive bacteria. Besides the establishment of expression systems relying on different inducible promoters (Bongers *et al.* 2005; Joseph *et al.* 2001; Kim *et al.* 1996), *B. subtilis* has been genetically engineered to improve the stability, the folding and the translocation of recombinant proteins (Thwaite *et al.* 2002; Vitikainen *et al.* 2005; Wu *et al.* 1991). However, cellular quality control systems like cell-wall associated proteases as well as the presence of multiple extracellular proteases have been shown to significantly reduce product yields (Lee *et al.* 2000). Moreover, a wide range of plasmids are not stably maintained in *B. subtilis* limiting its broad use as host for the production of recombinant proteins in industrial biotechnology.

1.4 The Molecular Limits of Recombinant Protein Production

The recombinant production of a protein in a given host represents a complex biological process. Numerous bottlenecks and limitations can influence the yield and/or the quality of the final product (Figure 1.5).

The construction of a functional gene expression system and the following transformation of a suitable expression host can already be a challenge. Especially, cloning of genes coding for harmfull products can be impaired if set under transcriptional control of promoters exhibiting basal activity. Therefore, the choice of a suitable promoter system is strongly dependent on the nature of the product and the desired rate of mRNA formation. Many different constitutive and inducible promoter systems are marketed for *E. coli*. The so-called T7 RNA polymerase dependent gene expression system shows exceptionally high transcriptional activity (Studier and Moffatt 1986). It is based on the highly processive DNA-dependent RNA polymerase of the

bacteriophage T7. *E. coli* engineered to recombinantly produce this viral polymerase can be used as host for the overexpression of heterologous genes whose transcription is under control of the T7 RNA polymerase dependent promoter. As a consequence mRNA levels can reach levels sufficient to saturate the protein synthesising machinery (Studier and Moffatt 1986).

In addition to the rate of transcription the structure of the mRNA as well as its stability can also provide a bottleneck in the formation of a target protein (Kudla *et al.* 2009). After their synthesis, mRNAs are prone to degradation by endogenous RNases. Hairpin structures within the 5'-untranslated region and transcription terminators represent stabilising elements which can prevent degradation and extend the half live of the corresponding mRNA. On the other hand, secondary structures near the ribosomal binding site reduce the efficiency of translational initiation. To date, no universally effective ribosomal binding site consensus sequence has been determined, but the complementarity of the ribosomal binding site and the 3'-end of the 16S rRNA seems to have a major effect on the efficiency of translational initiation in *B. subtilis* (Vellanoweth 1993).

Furthermore, limitations can influence the translation of mRNA into a growing polypeptide chain. Translation of mRNAs from homologous genes proceeds with high processivity. But genes of heterologous origin may be inefficiently translated if they display a codon usage bias which is divergent to the one of host organism.

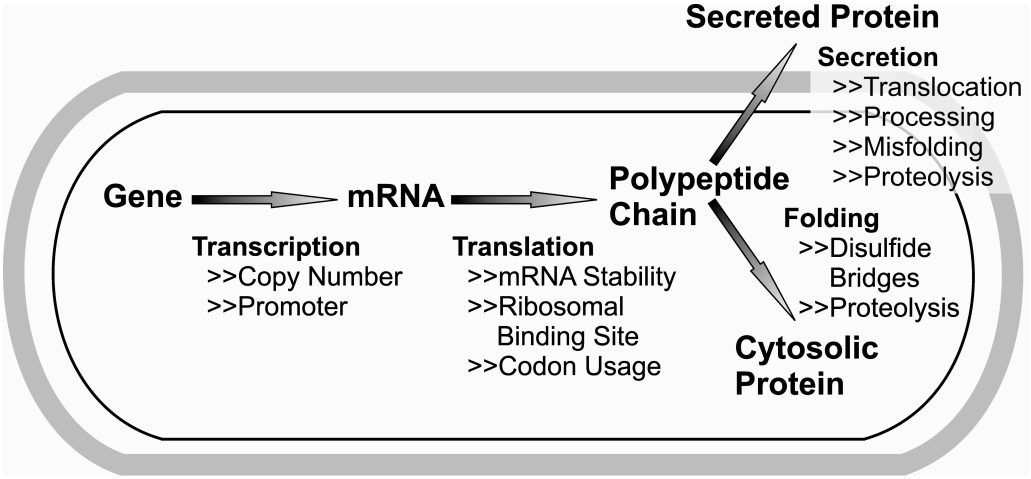

Figure 1.5: Bottlenecks in recombinant protein production. The biosynthesis of a target protein within a Gram-positive bacterium is schematically shown. Bottlenecks on the transcriptional and translational level which can influence the quantity and/or the quality of the recombinant protein are indicated. Moreover, possible limitations affiliated with the protein translocation and folding process are displayed.

Additional bottlenecks that limit the yield and/or the quality of the recombinant target protein appear posttranslationally. During protein biosynthesis the polypeptide chain

has to fold into its appropriate conformation. This process is either autocatalytic or assisted by molecular chaperones. High-level overproduction of a recombinant protein can result in insoluble, improperly folded aggregates. Moreover, proteins of heterologous origin are often subjected to proteolytic degradation by endogenous proteases. Targeting recombinant proteins to the subcellular space can circumvent problems associated with folding and/or proteoloytic degradation within the cell. Nevertheless, the translocation of a given protein is a complex process and potential bottlenecks on different levels have also been documented. It has been shown that processing of preproteins by signal peptidases can limit the release of mature proteins into the culture supernatant (Bolhuis *et al.* 1996; Malten *et al.* 2005b). Additionally, post-translocational protein misfolding as well as proteolytic degradation of secretory proteins by extracellular proteases can diminish the yield and/or the quality of the product (Bolhuis *et al.* 1999; Kontinen and Sarvas 1993).

Up to date, the choice of an adequate host for the economic production of a desired protein is a trial and error process. However, in the near future systems biology aims to mathematically model the complex dynamic process of recombinant protein production in a living organisms by integrating quantitative experimental data on the transcriptome, proteome and metabolome. A whole “Sonderforschungsbereich” entitled “From Gene to Product” (SFB578) at the Technische Universität Braunschweig is dedicted to this task using *Bacillus megaterium* as model organism.

1.5 *Bacillus megaterium*

The Gram-positive bacterium *B. megaterium* was discovered by De Bary in 1884 and is a mainly aerobic, spore-forming bacterium with an unusually large size (up to 100 times higher volume compared to *E. coli*) (Figure 1.6). It can be found in a variety of different habitats such as soil, seawater or sediments. Due to its size, it was optimally suited for studies of cell-structure, protein localisation, membranes and spore formation (Hrafnsdottir *et al.* 1997; McCool and Cannon 2001).

In the field of white biotechnology, *B. megaterium* has important characteristics for a broad application as host for the recombinant protein production in science and industry. Its ability to metabolise multiple carbon sources renders it capable to grow on cheap substrates (Vary *et al.* 2007). In 1990, von Tersch *et al.* developed a transformation procedure which allowed its genetic manipulation. Recombinant plasmids can be introduced into protoplasted *B. megaterium* cells (von Tersch and Robbins 1990) and stably maintained over many generations even without any selective pressure. Later on, Rygus and Hillen identified and characterised the

homologous xylose-inducible promoter offering the possibility for a regulated expression of recombinant genes (Rygus and Hillen 1991). Moreover, *B. megaterium* efficiently secretes proteins directly into the growth medium - an ability which is of essential industrial interest for the establishment of integrated biotechnological processes. Finally, the lack of extracellular alkaline proteases leads to an increased stability of secretory proteins within the culture supernatant (Malten *et al.* 2005a).

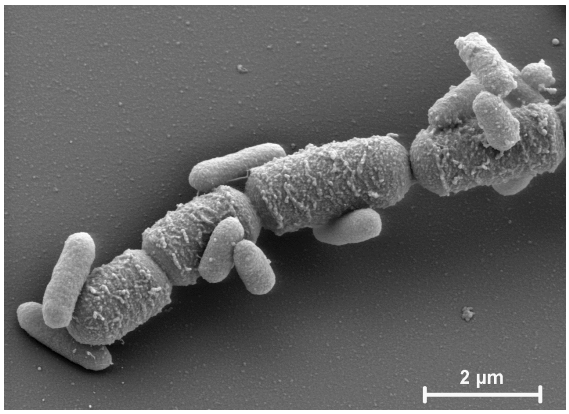

Figure 1.6: Electron microscopic image of *Bacillus megaterium* (big cells) with *Escherichia coli* (small cells) (Biedendieck *et al.* 2007b).

Up to now, various proteins of industrial interest have been produced with *B. megaterium*. Among those products are starch-modifying enzymes for the baking industry, like α- and β-amylases (David *et al.* 1987), penicillin amidases which are needed for the production of semisynthetic β-lactam antibiotics (Panbangred *et al.* 2000; Yang *et al.* 2001) and proteolytic enzymes employed in the leather tanning industry. Moreover, *B. megaterium* has been used for the production of fine chemicals like pyruvate, polyhydroxybutyrate (Hori *et al.* 2002) and vitamin B_{12} (Barg 2003; Barg *et al.* 2005).

1.5.1 Genome Sequence

Sequencing of whole genomes has markedly changed research in biological science and medicine. Since the first publication of a complete genome sequence of a living organism in 1995 (Fraser *et al.* 1995), many innovations in the field of genomics have been developed. Already in the year 2005, the genomes of more than 300 organisms have been sequenced by conventional Sanger sequencing methods (Rogers and Venter 2005). In the same year, Rothberg *et al.* succeeded in developing a pyrophosphate-based sequencing reaction (pyrosequencing) in picoliter-sized wells which enabled massively parallel sequencing (Margulies *et al.* 2005). This innovative

technology resulted in a 100 times faster analysis of whole genomes (25 million bases in a four hour period) than the conventional Sanger sequencing and capillary-based electrophoresis platform. Recently, even the successful sequencing of the whole human genome of James D. Watson by massively parallel sequencing within two months has been described (Wheeler *et al.* 2008).

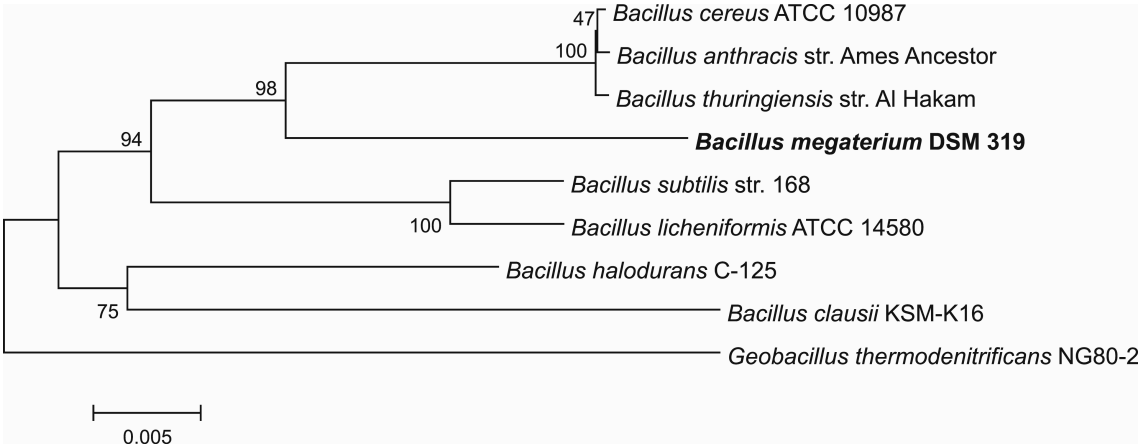

Figure 1.7: Phylogenetic tree of a subset of *Bacillus* species. The phylogenetic tree was calculated according to homologies in 16S rRNA sequences (Bunk, 2008, unpublished data).

Up to now, the genomes of 17 species of the genus *Bacillus*, including the most prominent representatives in industrial biotechnology, *B. subtilis* and *Bacillus licheniformis*, have been sequenced (as of 2008). Projects on sequencing of the *B. megaterium* genome of two different strains (DSM319 and QM B1551, respectively) started in parallel at the Technische Universität Braunschweig (Braunschweig, Germany) and at the J. Craig Venter Institute (Rockville, USA) in 2005. The genome sequence of the plasmidless *B. megaterium* strain DSM319 was solved by shotgun sequencing using a combination of conventional Sanger sequencing and high-throughput pyrosequencing in cooperation with the GATC-Biotech AG (Konstanz, Germany). The annotation of the first raw data started in 2005 (Hundertmark 2005). In 2008, last sequence gaps could be closed and the initial low coverage could be elevated to more than 20-fold. Altogether, the *B. megaterium* DSM319 genome has a size of ~5.2 Mbp with a GC-content of 38 %. Comparative analysis of 16S rRNA sequences showed that *B. megaterium* is closest related to *Bacillus thuringensis* (Figure 1.7).

1.5.2 Expression Systems

All commercially available expression systems for *B. megaterium* rely on the homologous xylose-inducible promoter. This promoter was described first in 1991 and

has been shown to have a high transcriptional activity (Rygus *et al.* 1991). It directs transcription of an operon coding for proteins responsible for xylose utilisation.

Detailed genetic analysis revealed that it is negatively regulated by a repressor protein which binds to two tandem overlapping operator sequences located downstream of the promoter. Thereby, it sterically prevents the DNA-dependent RNA polymerase to recognise its cognate -10 and -35 region. In the presence of xylose the repressor dissociates from the promoter. In this case, the RNA polymerase binds to the promoter and initiates transcription.

Based on the xylose-inducible promoter, Rygus and Hillen developed a plasmid-borne expression system for *B. megaterium*, enabling regulable, high-level production of recombinant proteins (Rygus and Hillen 1991). Diverse proteins of prokaryotic and eukaryotic origin were successfully produced intracellularly with this system. Later on, the basic expression system was further optimised. The multiple cloning site was extended to allow comfortable cloning of any gene of interest (Malten 2005) and coding sequences for affinity tags (StrepII-tag, His_6-tag) were introduced upstream and downstream of the multiple cloning site, respectively (Biedendieck 2007). The feasibility of the production of affinity-tagged recombinant proteins with *B. megaterium* and their purification was demonstrated by using the green fluorescent protein (GFP) as a model (Biedendieck *et al.* 2007c). Additionally, versatile secretion vectors were developed enabling the fusion of a gene of interest with the coding sequence of a signal peptide (Figure 1.8).

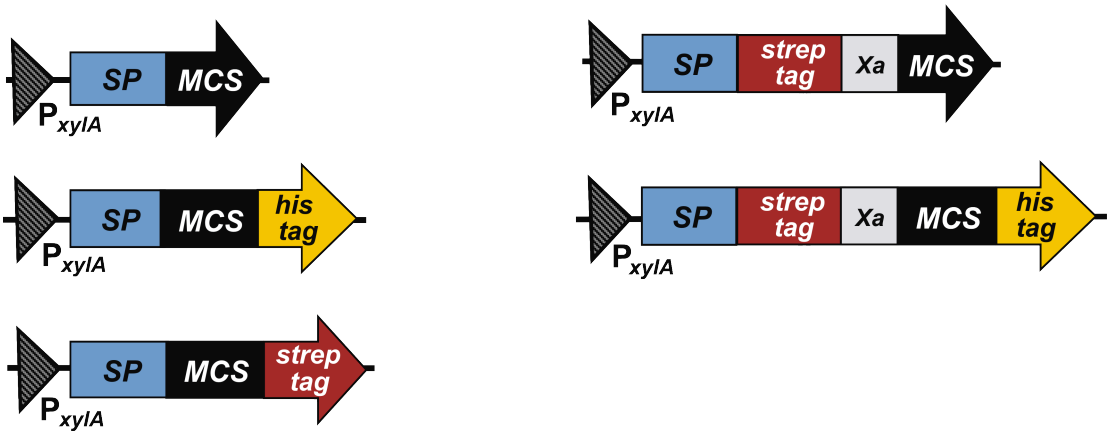

Figure 1.8: Vectors for the recombinant production of extracellular proteins with *Bacillus megaterium* (Biedendieck, 2007). Genes of interest can be cloned into the multiple cloning site (*MCS*) under transcriptional control of the xylose-inducible promoter (P_{xylA}). The secretion of recombinant proteins is enabled by the fusion of their corresponding genes with the signal peptide of the esterase LipA (*SP*). Coding regions for affinity tags (*strep tag* and *his tag*, respectively) allow for their incorporation into a protein of interest. The recognition site for the factor Xa protease (*Xa*) can be used for the removal of the N-terminally located affinity tag.

All secretion vectors rely on the signal peptide of the homologous esterase LipA which directs proteins to the environment by the secretion-dependent pathway (SEC-pathway) (Malten *et al.* 2006). This pathway is most prominently used by Gram-positive bacteria to translocate endogenous proteins to the subcellular milieu (Antelmann *et al.* 2001). Using the signal peptide of LipA as export-signal, a levansucrase and a hydrolase were successfully produced and translocated to the culture medium by *B. megaterium* (Biedendieck *et al.* 2007a; Yang *et al.* 2007).

1.6 Genetic Engineering of Microbial Host Organisms

The selective inactivation of chromosomal genes is essential for the rational optimisation of microbial hosts in the sense of eliminating bottlenecks and thereby elevating the quality and quantity of recombinant proteins (chapter 1.4). Recently, the company Gene Bridges (Dresden, Germany) introduced a convenient method which allows for the precise deletion and/or insertion of genes on the *E. coli* chromosome within a week. It relies on a viral exopolymerase and a single strand binding protein which mediates recombination of a given DNA fragment with the chromosomal DNA by homologous sequence elements (Muyrers *et al.* 2001). Attempts to adopt this genetic engineering system to *B. megaterium* did not succeed (Norden 2006).
B. subtilis on the other hand is naturally highly amenable to genetic engineering. In the early 1960's, Spizizen and Anagnostopoulos discovered the ability of *B. subtilis* to actively take up extracellular DNA and to incorporate it into its genome (Anagnostopoulos and Spizizen 1961; Spizizen 1958). This property was defined as natural competence for transformation and has been extensively used for the rational modification of *B. subtilis* by targeted gene-knockouts and -knockins.

1.6.1 Natural Competence in *Bacillus subtilis*

Since its discovery, detailed studies on natural competence in *B. subtilis* led to the identification of many genes involved in its regulation and to the detection of genes coding for proteins of the DNA translocation machinery. Localisation studies showed that the DNA uptake pore is mainly localised at the cell poles and plays a central role during transformation (Hahn *et al.* 2005). The whole transformation process of competent *B. subtilis* cells involves I) binding of the DNA, II) transport across the membrane, III) protection of incoming DNA and IV) recombination or reconstitution (Figure 1.9).

Binding of Exogenous DNA

The first step in the acquisition of exogenous DNA comprises its binding to the cells. Due to the Gram-positive nature of *B. subtilis*, this process is very complex because of the presence of a multilayer peptidoglycan-containing cell wall. To facilitate binding of extracellular DNA to competent *B. subtilis* cells, the bacteria produce proteins which are encoded by the chromosomal *comG* operon. This operon comprises seven genes (ComGA-ComGG) which are all essential for the formation of a pseudopilus (Chen *et al.* 2006). Transcription of *comGC*, *comGD*, *comGE*, *comGF* and *comGG* leads to the formation of prepilins which integrate into the cytoplasmatic membrane. After their processing by the peptidase ComC, they translocate to the exterior of the membrane (Chung *et al.* 1998). The formation of a supramolecular pseudopilus thereby modulates the cell wall in such a way that the DNA gains access to the receptor protein ComEA (Provvedi and Dubnau 1999).

Transport of DNA Across the Membrane

ComEC plays a central role in the transport of DNA across the cell membrane. It is thought to form an aqueous pore through which the DNA is transported into the cell by proton motive force (Draskovic and Dubnau 2005). Additionally, the membrane associated protein ComFA, structurally similar to ATP-dependent helicases, is suggested to act as the motor protein for DNA transport (Londono-Vallejo and Dubnau 1993). Moreover, its helicase activity may be responsible for the unwinding of double stranded incoming DNA. During transformation, only single stranded DNA is transported into the cell. The non-transforming strand is degraded by a yet unidentified membrane associated nuclease. The degradation products are released into the medium.

Protection of Incoming DNA

Incoming single stranded DNA has to be protected against degradation by host DNases. Competence-induced single strand DNA binding proteins (ssb proteins) mediate a stabilising effect against the action of these nucleases (Eisenstadt *et al.* 1975). Additionally, RecA, also known for its central role in DNA repair mechanisms, is thought to form transient nucleoprotein filaments which connect the polar site of DNA uptake with the host-cell chromosome where integration takes place (Kidane and Graumann 2005).

Recombination and Reconstitution

The integration of exogenous DNA into the host chromosome strongly depends on the nature of the DNA and the degree of homology. RecA mediates the recombination between homologous regions of the single stranded DNA and the host chromosome. It thereby enables the stable integration of new genetic elements into the genome of *B. subtilis*.

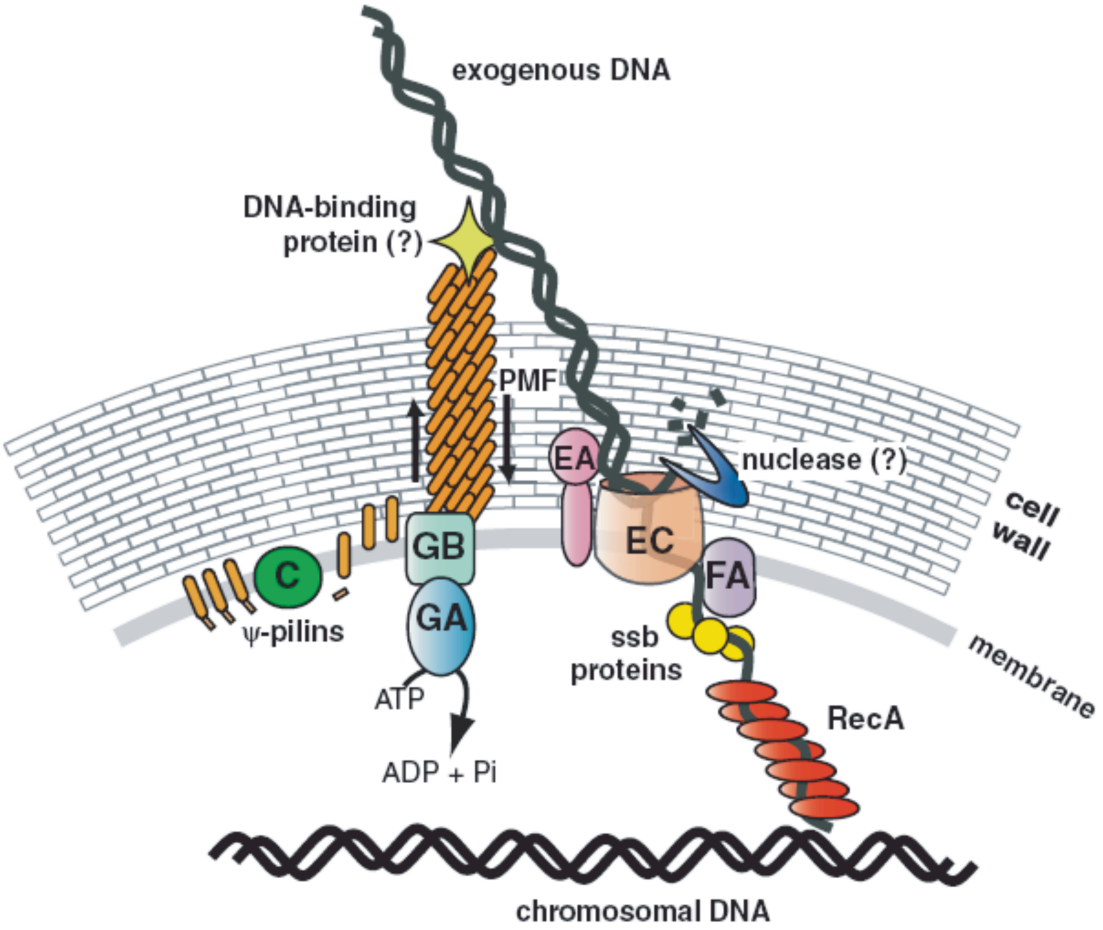

Figure 1.9: Uptake of exogenous DNA in *Bacillus subtilis* (modified according to (Chen *et al.* 2005)). Prepilins are processed by ComC and translocated to the outer surface of the cell membrane. Due to the NTPase activity of ComGA and with the help of the membrane protein ComGB, the main pilin ComGC as well as the minor pilins ComGD, ComGE and ComGG are assembled into a pseudopilus. The pseudopilus enables the receptor protein ComEA to get access to exogenous DNA. One strand of the DNA is degraded by a yet unidentified nuclease. The other strand is transported through an aqueous pore (ComEC) into the cell by proton motive force (PMF). The helicase/DNA translocase ComFA as well as single strand DNA binding proteins (ssb proteins) are involved in the translocation and stabilisation of the incoming DNA. RecA forms a filament around the ssDNA and mediates search for homology with chromosomal DNA.

Regulation of Competence Development

The development of competence leads to dramatic changes in the physiology: Competent cells cease DNA-replication, block the synthesis of macromolecules and are increasingly sensitive towards mutagens (Dubnau 1991). Due to this dramatic impact on the long-term survival of *B. subtilis*, the development of competence is tightly regulated.

The regulation of competence development is a complex process which depends on numerous factors like the cell density of the culture and the availability of nutrients. ComK represents the central transcription factor in competence development (van Sinderen *et al.* 1995). It is a small protein, 192 amino acids in size and recognises a specific sequence named K-box. This motif is composed of two palindromic sequences ($A_4N_5T_4$), the so-called AT-box. ComK is believed to bind as a dimer, thereby bending the DNA and mediating a stabilisation of the RNA polymerase binding (Susanna *et al.* 2004).

The formation of ComK is controlled by a complex cellular network which involves quorum sensing molecules and autoregulatory elements (Figure 1.10). Transcription of *comK* is repressed by AbrB, CodY and Rok. On the other hand ComK can activate its own transcription by binding to a K-box located upstream of *comK*. Additionally, the activity of ComK is posttranslationally affected by the action of a proteolytic complex consisting of MecA and the protease ClpCP (Figure 1.). Further, high cell density is a prerequiste for optimal competence development and at least two independent quorum sensing pathways are involved in the regulation of ComS formation. ComS is a positive regulator in competence development as it liberates ComK from the proteolytic complex.

Microarray analysis revealed that ComK directly or indirectly activates expression of more than 100 genes (Berka *et al.* 2002). Its direct involvement in the transcription of genes which are essential for the binding, the translocation and the integration of exogenous DNA into the host chromosome has been proven my multiple studies (Berka *et al.* 2002; van Sinderen *et al.* 1995).

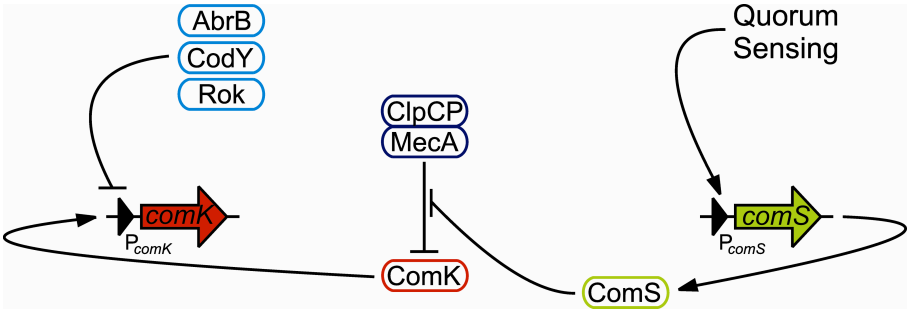

Figure 1.10: Regulation of competence development in *Bacillus subtilis*. Arrows denote upregulation and blunt ends denote downregulation. During exponential growth the level of ComK is low due to its efficient proteolytic degradation by the proteolytic complex consisting of MecA and the protease ClpCP and due to its transcriptional repression via the regulators AbrB, CodY and Rok. High cell densities lead to the transcriptional activation of P_{comS} due to quorum sensing mechanisms. The subsequent formation of ComS leads to the inhibition of the proteolytic complex and thereby to an increased stability of ComK. ComK activates its own transcription via interaction with P_{comK}. This autoregulatory loop ensures rapid formation of ComK if its cellular levels reach a certain threshold.

1.6.2 Natural Competence in the Genus *Bacillus*

B. subtilis was the first organism of the genus *Bacillus* which was known to develop natural competence for DNA binding and uptake. Comparative genomics revealed that genes coding for orthologues involved in the development of natural competence in *B. subtilis*, seem to be present in almost all yet sequenced *Bacillus* species (Kovacs *et al.* 2009). However, up to now only few species have been reported to become naturally competent. Among those are *B. licheniformis* and *Bacillus amyloliquefaciens* which have been shown to take up exogenous DNA under defined growth conditions in specific media (Koumoutsi *et al.* 2004; Thorne and Stull 1966). In 2008, Kuipers *et al.* demonstrated that the development of natural competence could be induced in *B. cereus* by overproducing the central transcription factor ComK of *B. subtilis* (Mironczuk *et al.* 2008). For the first time it was shown that ComK of *B. subtilis* is functional in *B. cereus* and leads to the transcription of genes involved in the formation of a functional DNA uptake pore.

Natural competence enables multiple biotechnological applications like high-throughput gene-knockouts and -knockins and is therefore, more than 40 years of its discovery, still in the focus of applied science. Currently, industrial companies as well as academic institutions try to establish natural competence for DNA binding and uptake in industrially important *Bacillus* species in order to broaden their biotechnological amenabilities (personal communication with Prof. Dr. Meinhardt, University Münster, Germany and Prof. Dr. Kuipers, University Groningen, Netherlands).

1.7 Aim of This Study

The research presented in this thesis aimed to broaden the applicability of *B. megaterium* as alternative host for the high-level production of recombinant proteins in science and industry.

The first part deals with the process of protein biosynthesis which was to be analysed and improved. The process of transcription had to be enhanced by establishing an alternative gene expression system which relies on the RNA-polymerase of the bacteriophage T7. This system should be evaluated for the recombinant production of intra- as well as extracellular proteins. The influence of individual codons on the efficiency of the translation from mRNA into the respective protein was to be evaluated and limiting tRNAs had to be determined. Moreover, the feasability of the coexpression of a rate limiting tRNA with respect to an elevated formation of recombinant proteins had to be tested. A detailed analysis regarding the capability of *B. megaterium* to develop natural competence for genetic transformation was of interest. Insights into this process of bacterial differentiation should broaden the genetic amenability of industrially important *B. megaterium*.

The final part of this thesis aimed to the production and secretion of two different eukaryotic proteins of industrial importance. This work was carried out in cooperation with the BASF SE (Ludwigshafen, Germany). Bottlenecks limiting protein formation had to be identified to improve the yield in recombinant protein systematically.

2 MATERIALS AND METHODS

2.1 Instruments, Chemicals and Kits

2.1.1 Instruments

All instruments and technical devices used in this study are listed in Table 2.1.

Table 2.1: Instruments and technical devices.

Instrument	Product Specification	Manufacturer
Agarose Gel Documentation	GelDoc	Bio-Rad
Agarose Gel Electrophoresis	Agagel	Biometra
Autoclav	LVSY 50/70	Zirbus
Blotting	Trans Blot apparatus (semi dry transfer cell)	Bio-Rad
Centrifuges	Biofuge pica	Heraeus
	Centrifuge 5403	Eppendorf
	Centrifuge 5415 C	Eppendorf
	RC 5B Plus	Sorvall
	SpeedVac SPD 110B with Refrigerated Vapour Trap RVT400	Savant
Contact Angle Measurement System	OCA 15+	DataPhysics Instruments
Digital Camera	Cyber shot	Sony
Dismembrator	Mikro-Dismembrator S	B. Braun Biotech International
DNA Sequencing	ABI PRISM™ 310 Genetic Analyser	Applied Biosystems
ELISA Reader	Fusion	Perkin Elmer
Film Processor	Optimax	Protec
Flow Cytometer	EPICS XL-MCL	Beckman Coulter
Fluorescence Microscope	Axiovert 200M with AxioCam HR/ApoTome and Illuminator HB0/HAL100	Carl Zeiss
Gradient Cycler	Tgradient	Biometra
Luminescence Spectrometer	LS50B	PerkinElmer
pH Determination	pH-Meter C 6840 B	Schott
Pipettes	Reference	Eppendorf
Scales	SBA 52	Scaltec
	HC 52	Mettler
SDS-PAGE	Mini Protean II	Bio-Rad
Shaker	Bench Top Shaker, TR	Infors AG
Spectrophotometer	Ultrospec 200	GE Healthcare
Thermocycler	Tpersonal	Biometra
Thermomixer	Thermomixer compact	Eppendorf
Transilluminator	Flu-O-blu	Biozym
UV Crosslinker	UV Stratalinker 2400	Stratagene

Table 2.1 (continued): Instruments and technical devices.

Instrument	Product Specification	Manufacturer
Water Bath	W200	Memmert
Water Bath Shaker	Aquatron	Infors AG
Ultra Pure Water System	Synthesis A10	Millipore
96-Well Plate Shaker	Thermo Shaker PST-60HL-4	Lab4you

2.1.2 Chemicals and Reagents

All chemicals and reagents used in this study are listed in Table 2.2. Additional chemicals and reagents not listed were purchased from Difco, Fluka, GE Healthcare, Merck, Oxoid, Riedel-de-Häen, Roth and Sigma-Aldrich.

Table 2.2: Chemicals and reagents.

Product	Manufacturer
Affinity Material for Protein Purification	
Chelating Sepharose FF	GE Healthcare
Antibodies	
mc-mouse Anti-His_6	GE Healthcare
pc-goat α mouse IgG-Fc-alkaline phosphatase	Sigma-Aldrich
pc-goat a mouse IgG (H+L)-horseradish peroxidase (HRP)	Pierce
Blotting Membrane	
Roti-PVDF Transfer Membrane	Roth
Blotting Paper	
Gel-Blotting-Paper (150 mm)	Neolab
Chemiluminescent Substrate	
CDP-*Star*	Roche Diagnostics
SuperSignal West Pico Mouse IgG Detection Kit	Pierce
DNA Purification	
QIAquick Gel Extraction Kit	Qiagen
QIAprep Spin Miniprep Kit	Qiagen
QIAquick PCR Purification Kit	Qiagen
DNA Size Standards	
GeneRule DNA Ladder Mix	Fermentas
MassRuler™ DNA Ladder Mix	Fermentas
Enzymes for Molecular Biological Applications	Biotherm Finnzymes GE Healthcare GeneCraft Fermentas Merck New England BioLabs Promega
Fluorescent DNA Stain	
GelStar Nucleic Acid Gel Stain	Lonza
Oligonucleotides	Biomers Metabion

Table 2.2 (continued): Chemicals and reagents.

Product	Manufacturer
RNA Labeling	
DIG RNA Labeling Kit	Roche Diagnostics
RNA Size Standard	Roche Diagnostics
Protein Size Standards	
PageRuler Prestained Protein Ladder	Fermentas
Protein Molecular Weight Marker	Fermentas
Sterile Filter	Millipore

2.1.3 Bacterial Strains and Plasmids

All bacterial strains and plasmids used for this study are listed in Table 2.3 and 2.4.

Table 2.3: Bacterial strains.

Name	Description	Reference/Source
Bacillus megaterium		
DSM319	Wild type	DSMZ, Braunschweig, Germany
MS941	Mutant of DSM319, Δ*nprM*	(Wittchen and Meinhardt 1995)
PV361	Plasmid-less derivative of QM B1551	(Sussman *et al.* 1988)
WH323	Mutant of WH320, *xylA1-spoVG-lacZ*	(Rygus and Hillen 1992)
YYBm1	Mutant of DSM319, Δ*nprM*, Δ*xylA*, Ery^r	(Yang *et al.* 2006)
Escherichia coli		
DH10B	F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ (*ara*, *leu*)7697 *gal*U *gal*K λ- *rps*L *nup*G /pMON14272 / pMON7124	Invitrogen
XL10-Gold	Tet^{r}Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F´ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^r) Amy Cam^r]	Stratagene

Table 2.4: Plasmids.

Plasmid	Description	Reference/Source
Codon Usage and Recombinant Protein Production in *Bacillus megaterium*		
pMM1522	BsrGI restriction site inserted into pMM1520 upstream of the start codon of the open reading frame including the MCS	(Biedendieck 2007)
pYZ5	Truncated form of pYZ11 (Kunnimalaiyaan *et al.* 2001), shuttle vector for cloning in *E. coli* (Ap^r) and replication in *B. megaterium* (Cm^r) containing 1.1 kb fragment of pBM100	Vary, personal gift
pRBBm34	Shuttle vector for the recombinant production of GFP in *B. megaterium*	(Biedendieck *et al.* 2007b)
pMGBm63	*gfp* fused to a cluster of 4 CGG codons (encoding arginine) cloned into *Spe*I and *Eag*I of pMM1522	This study
pMGBm64	*gfp* fused to a cluster of 4 CGT codons (encoding arginine) cloned into *Spe*I and *Eag*I of pMM1522	This study
pMGBm65	*gfp* fused to a cluster of 4 CTC codons (encoding leucine) cloned into *Spe*I and *Eag*I of pMM1522	This study
pMGBm66	*gfp* fused to a cluster of 4 TTA codons (encoding leucine) cloned into *Spe*I and *Eag*I of pMM1522	This study
pMGBm67	*gfp* fused to a cluster of 4 TCA codons (encoding serine) cloned into *Spe*I and *Eag*I of pMM1522	This study
pMGBm68	*gfp* fused to a cluster of 4 TCC codons (encoding serine) cloned into *Spe*I and *Eag*I of pMM1522	This study
pMGBm70	*gfp* fused to a cluster of 4 GGG codons (encoding glycine) cloned into *Spe*I and *Eag*I of pMM1522	This study
pMGBm71	*gfp* fused to a cluster of 4 GGA codons (encoding glycine) cloned into *Spe*I and *Eag*I of pMM1522	This study
pMGBm72	*gfp* fused to a cluster of 4 GCC codons (encoding alanine) cloned into *Spe*I and *Eag*I of pMM1522	This study
pMGBm73	*gfp* fused to a cluster of 4 GCT codons (encoding alanine) cloned into *Spe*I and *Eag*I of pMM1522	This study
pMGBm78	*gfp* fused to a cluster of 4 AGG codons (encoding arginine) cloned into *Spe*I and *Eag*I of pMM1522	This study
pMGBm96	*gfp* fused to a cluster of 4 ACC codons (encoding threonine) cloned into *Spe*I and *Eag*I of pMM1522	This study
pMGBm97	*gfp* fused to a cluster of 4 ACA codons (encoding threonine) cloned into *Spe*I and *Eag*I of pMM1522	This study
pRBBm40	*repU-oriU* of pMM1520 cloned into the *Aat*II site of pMUTIN-gfp+	(Biedendieck 2007)
pYZ5-P_{spac} alternative name: pMGBm86	P_{spac} of pRBBm40 cloned into *Sac*I and *Xma*I of pYZ5	This study
ptRNA $^{Thr}_{GGU}$ alternative name: pCBBm10	Putative tRNA $^{Thr}_{GGU}$ of *B. megaterium* PV361 including its assumed promoter region cloned into *Sac*I and *Xma*I of pYZ5	This study

Table 2.4 (continued): Plasmids.

Plasmid	Description	Reference/Source
T7 RNA Polymerase Dependent Gene Expression in *Bacillus megaterium*		
pMM1520	Shuttle vector for cloning in *E. coli* (Ap^r) and gene expression under xylose control in *B. megaterium* (Tc^r)	(Malten 2005)
pStop1622	Shuttle vector for cloning in *E. coli* (Ap^r) and gene expression in *B. megaterium* (Tc^r)	(Biedendieck *et al.* 2007c)
pRBBm34	Shuttle vector for the recombinant production of GFP in *B. megaterium*	(Biedendieck *et al.* 2007b)
pRBBm15	Shuttle vector for the recombinant production and secretion of levansucrase LevΔ773 in *B. megaterium*	(Malten *et al.* 2006)
pYZ5	Truncated form of pYZ11 (Kunnimalaiyaan *et al.* 2001), shuttle vector for cloning in *E. coli* (Ap^r) and replication in *B. megaterium* (Cm^r) containing 1.1 kb fragment of pBM100	Vary, personal gift
pMGBm19	*xylR*-P_{xylA}-*xylA'*-MCS cloned into *Bam*HI and *Nar*I of pYZ5	This study
pStop1622-Pϕ10	ϕ10 promoter cloned into *Afl*II and *Bsr*GI of pStop1622	This study
pP_{T7}	ϕ10 terminator cloned into *Age*I and *Sph*I of pStop1622-Pϕ10	This study
pT7-RNAP	*t7-rnap* fused to 4 x GGA base triplets at its 5'-end cloned in *Spe*I and *Xho*I of pMGBm19	This study
pP_{T7}-GFP	*gfp* cloned in *Spe*I and *Sph*I of pP_{T7}	This study
pP_{T7}-Lev	sp_{lipA}-*levΔ773*-his_6-*tag* cloned in *Bsr*GI and *Sac*I of pP_{T7}	This study
***Bacillus megaterium* – an Alternative Host for the Production of Recombinant Proteins of Eukaryotic Origin**		
pPCR-Script051172	Codon optimised *kbd b* encoding a N-terminal His_6-tag and a linker fragment cloned into *Kpn*I and *Sac*I of pPCR-Script (Stratagene, La Jolla, USA)	Geneart AG
0605024pPCR-Script	Codon optimised *dewA* including its coding region for the native signal peptide and for a C-terminal His_6-tag cloned into *Kpn*I and *Sac*I of pPCR-Script (Stratagene, La Jolla, USA)	Geneart AG
pStop1622	Shuttle vector for cloning in *E. coli* (Ap^r) and gene expression in *B. megaterium* (Tc^r)	(Biedendieck *et al.* 2007c)
pMM1525	pMM1522 derivative containing the DNA sequence coding for the signal peptide of the *B. megaterium* extracellular esterase LipA (SP_{lipA})	(Malten *et al.* 2006)
pHis1525	pADBm5 derivative; vector for the secretion of recombinant C-terminal His_6-tagged proteins in *B. megaterium*	(Biedendieck 2007)
pNewHis1525 alternative name: pMGBm9	pHis1525 derivative with altered sequence between *Sph*I and *Age*I coding for a C-terminal His_6-tag	This study

Table 2.4 (continued): Plasmids.

Plasmid	Description	Reference/Source
pP_{xylA}-kbdb-his_6^* alternative name: pMGBm11	Codon optimised *kbd b* including the N-terminal linker fragment cloned into *Sph*I and *Age*I of pNewHis1525	This study
pP_{xylA}-kbdb-his_6 alternative name: pMGBm12	Truncated derivative of pP_{xylA}-kbdb-his_6^* (loss of 30 bp *Nar*I-Fragment)	This study
pP_{xylA}-his_6-kbdb* alternative name: pMGBm25	Codon optimised *kbd b* including the coding region for a N-terminal His_6-tag cloned into *Sac*I and *Sph*I of pMM1525	This study
pP_{xylA}-his_6-kbdb alternative name: pMGBm26	Truncated derivative of pP_{xylA}-his_6-kbdb* (loss of 30 bp *Nar*I-Fragment)	This study
pP_{xylA}-sp_{lipA}-dewA-his_6^* Alternative name: pMGBm46	Codon optimised *dewA* without the coding region of the native signal peptide and with the one for a C-terminal His_6-tag cloned into *Sac*I and *Sph*I of pMM1525	This study
pP_{xylA}-sp_{lipA}-dewA-his_6 Alternative name: pMGBm47	Truncated derivative of pP_{xylA}-sp_{lipA}-dewA-his_6^* (loss of 30 bp *Nar*I-Fragment)	This study
pP_{xylA}-sp_{native}-dewA-his_6 Alternative name: pMGBm52	Codon optimised *dewA* with the coding region of the native signal peptide and with the one for a C-terminal His_6-tag cloned into *Bsr*GI and *Sph*I of pMM1525	This study
Development of Natural Competence in *Bacillus megaterium*		
pMM1622	Equal to pMM1522, lacking 855 bp between *Afl*II restriction sites	Biedendieck, personal gift
pStop1622	Shuttle vector for cloning in *E. coli* (Ap^r) and gene expression in *B. megaterium* (Tc^r)	(Biedendieck *et al.* 2007c)
pN-His-TEV1622	pStop1622 derivative; vector for the intracellular production of N-terminally His_6-tagged proteins in *B. megaterium*	(Biedendieck 2007)
pYZ5	Truncated form of pYZ11 (Kunnimalaiyaan *et al.* 2001), shuttle vector for cloning in *E. coli* (Ap^r) and replication in *B. megaterium* (Cm^r) containing 1.1 kb fragment of pBM100	Vary, personal gift
pSKE194	Shuttle vector for *E. coli* (Ap^r) and *B. megaterium* (Ery^r)	(Nahrstedt *et al.* 2005)
$pNWcomK_{Bsu}$	Vector for the recombinant expression of $comK_{Bsu}$ in *Bacilli*	(Mironczuk *et al.* 2008)

Table 2.4 (continued): Plasmids.

Plasmid	Description	Reference/Source
pRBBm34	Shuttle vector for the recombinant production of GFP in *B. megaterium*	(Biedendieck *et al.* 2007b)
pDFHis-comK_{Bmeg}	*comK_{Bmeg}* cloned into *Bgl*II and *Eag*I of pN-His-TEV1622	This study
pDFcomK_{Bmeg}	*comK_{Bmeg}* cloned into *Bsr*GI and *Eag*I of pStop1622	This study
pDFcomK_{Bsu}	*comK_{Bsu}* cloned into *Bsr*GI and *Sph*I of pStop1622	This study
pDFgfp	*gfp* cloned into *Kpn*I and *Eco*RI of pYZ5	This study
pDFrecA-gfp	Putative promoter region of *recA* from *B. megaterium* DSM319 cloned into *Kpn*I and *Xba*I of pDFgfp	This study
pDFcomG-gfp	Putative promoter region of the *comG* operon from *B. megaterium* DSM319 cloned into *Age*I and *Kpn*I of pDFgfp	This study

2.2 Growth Media and Media Additives

2.2.1 Media for *Escherichia coli* and *Bacillus megaterium*

As a standard medium for growth of all bacterial strains, Luria Bertani (LB) medium (Sambrook *et al.*, 1999) was used unless indicated otherwise. For solid media, 1.5 % (w/v) agar-agar was added prior to sterilisation.

Luria-Bertani Broth (LB Medium)	
Tryptone	1.0 % (w/v)
Yeast Extract	0.5 % (w/v)
NaCl	0.5 % (w/v)

Tryptone-Yeast Extract Broth (TY Medium)	
Tryptone	1.0 % (w/v)
Yeast Extract	0.5 % (w/v)
NaCl	1.0 % (w/v)

Recombinant protein production experiments were performed in LB medium, TB medium or A5+4 medium.

Terrific Broth (TB Medium)		
Tryptone	1.2	% (w/v)
Yeast Extract	2.4	% (w/v)
Glycerol	0.4	% (v/v)
Casamino Acids	0.1	% (w/v)
After autocalving, addition of:		
KH_2PO_4	17.0	mM
K_2HPO_4	72.0	mM
D-Glucose	50.0	mM

A5+4 Medium		
D-Glucose	166.5	mM
$(NH_4)SO_4$	15.1	mM
$MgSO_4$ x 7 H_2O	1.2	mM
KH_2PO_4	25.9	mM
Na_2HPO_4 x 2 H_2O	40.8	mM
$MnCl_2$ x H_2O	278.0	µM
$CaCl_2$ x 7 H_2O	410.8	µM
$FeSO_4$ x 7 H_2O	9.0	µM
$(NH_4)_6Mo_7O_{24}$ x H_2O	2.1	µM
$CoCl_2$ x 6 H_2O	10.5	µM
Yeast Extract	0.4	% (w/v)

Experiments regarding the development of natural competence for genetic transformation were either performed in LB medium or minimal medium.

Minimal Medium		
Casamino Acids	0.02	% (w/v)
K_2HPO_4	62.0	mM
KH_2PO_4	44.0	mM
$(NH_4)_2SO_4$	15.0	mM
Na Citrate	6.5	mM
$MgSO_4$	800.0	µM
L-Tryptophane	100.0	µM
Fructose	27.8	mM
Fructose was sterilised by filtration (pore size 0.2 µm).		

2.2.2 Media Additives

Antibiotics and other additives were prepared as concentrated stock solutions, sterilised by filtration and added into the medium after autoclaving. Solutes and concentrations are summarised in Table 2.5.

Table 2.5: Media additives. Final antibiotic concentrations marked with "*" were applied in experiments aimed to analyse the development of natural competence for genetic transformation in individual *B. megaterium* plasmid strains.

Additive	Bacterial Strain	Stock Solution	Final Concentration
Carbenicillin	*E. coli*	100 mg ml^{-1} in dH_2O	100 µg ml^{-1}
Chloramphenicol	*B. megaterium*	4.5 mg ml^{-1} in 70 % (v/v) ethanol	2.5* µg ml^{-1} 4.5 µg ml^{-1}
Erythromycin	*B. megaterium*	5 mg ml^{-1} in 70 % (v/v) ethanol	0.5 µg ml^{-1} 5.0 µg ml^{-1}
Rifampicin	*B. megaterium*	34 mg ml^{-1} in methanol	200 µg ml^{-1}
Tetracyclin	*B. megaterium*	5 mg ml^{-1} in 70 % (v/v) ethanol	2.5* µg ml^{-1} 10.0 µg ml^{-1}
Xylose	*B. megaterium*	50 % (w/v)	0.5 – 3.0 % (w/v)

2.3 Microbiological Techniques

2.3.1 Sterilisation

All media were vapor sterilised at 121°C and 1 bar positive pressure for 20 min. Other substances and solutions were either vapor sterilised or, if temperature sensitive, sterilised by filtration (pore width 0.2 µm).

2.3.2 Plate Cultures

Bacteria were plated directly from a glycerol stock onto a LB medium agar plate. When required, antibiotics or other additives were added. Agar plates were incubated overnight at 30°C or 37°C.

2.3.3 Liquid Cultures of *Escherichia coli*

Liquid cultures were inoculated using a single colony from a LB medium agar plate. The medium was supplemented with the appropriate antibiotics when required. Cultures were shaken at 200 rpm in test tubes or baffled flasks at 37°C. The incubation times varied depending on the desired optical densities.

2.3.4 Liquid Cultures of *Bacillus megaterium*

Liquid cultures in LB medium were inoculated using a single colony from a LB medium agar plate for pre-cultures. The medium was supplemented with the appropriate

antibiotics when required. Cultivations were performed at 37°C in baffled flasks at 100 rpm (Aquatron, Infors AG, Switzerland) for approximately 14 h.
Liquid main cultures were inoculated at the ratio of 1:100 from the pre-cultures. Cultivations were performed at 30°C or at 37°C in baffled flasks at 250 rpm (Aquatron, Infors AG, Switzerland).

2.3.5 Liquid Cultures of *Bacillus megaterium* in 96-Well Plates

Pre-cultures of *B. megaterium* were prepared as described above (chapter 2.3.4). Main cultures in minimal medium were inoculated with the pre-cultures to a final OD_{600} of 0.15. The medium was supplemented with appropriate antibiotics. The cultivation of the main cultures was performed in black 96-well plates with optical bottom (Nunc, Roskilde, Dänemark) at 37°C and 1,000 rpm (Thermo Shaker PST-60HL-4, Lab4you). 180 µl of total culture volume was applied per well.

2.3.6 Determination of Cell Density

The cell densities of liquid cultures were determined by measuring the OD at a wavelength of 578 nm or 600 nm. For cell densities with an OD>0.8, dilutions of the cell culture broth were prepared. An OD_{578} of 1 corresponded to approximately 1 x 10^9 cells per ml.

2.3.7 Storage of Bacteria

Strains were kept on LB medium agar plates at 4°C for up to 10 days (*B. megaterium*) or up to 4 weeks (*E. coli*). For long-term storage of bacteria, glycerol cultures were prepared. For this purpose, *B. megaterium* cultures were incubated for approximately 14 h and 100 rpm (Aquatron, Infors, Switzerland) at 37°C. A culture volume of 650 µl was gently mixed with 350 µl of 87 % (w/v) glycerol. Stocks were immediately frozen and stored at -80°C.

2.4 Molecular Biology Techniques

2.4.1 Preparation and Transformation of Chemically Competent *Escherichia coli*

5 ml LB medium were inoculated with a single colony of *E. coli* DH10B or *E. coli* XL10-Gold and cultivated for approximately 14 h at 37°C. 1 ml of this culture was used to inoculate 100 ml of LB medium. The bacteria were incubated at 37°C and 200 rpm (Aquatron, Infors AG, Switzerland) in baffled flasks until the culture reached an OD_{578} of 0.8. After cooling on ice water for 10 min, the cells were harvested by centrifugation (2,600 x g, 10 min, 4°C). The cells were suspended in 10 ml of ice-cold $CaCl_2$ solution and incubated on ice for 15 min. After centrifugation (2,600 x g; 10 min; 4°C), the cells were suspended in 1 ml of ice-cold $CaCl_2$ solution. The competent cells were either used directly for transformation or were stored at -80°C.

Transformation of chemically competent cells was applied as standard transformation procedure. 50 µl of $CaCl_2$-competent *E. coli* were mixed with 1-2 µl of DNA solution (50 µg ml^{-1}), incubated on ice for 20 min and subjected to a heat shock for 2 min at 42°C. Immediately thereafter, the sample was cooled on ice for 2 min. Afterwards, 1 ml of preheated LB medium was added and cells were incubated at 37°C for 1 h. Depending on the expected transformation efficiency, different volumes were streaked onto LB medium agar plates containing the appropriate antibiotics. Incubation was performed overnight at 37°C.

$CaCl_2$ Solution

$CaCl_2$	100.0 mM
Glycerol	10.0 % (w/v)

2.4.2 Preparation and Transformation of *Bacillus megaterium* Protoplasts

50 ml of LB medium were inoculated with an individual *B. megaterium* colony and grown for approximately 14 h at 37°C and 100 rpm (Aquatron, Infors AG, Switzerland) in baffled flasks. 1 ml of this culture was used to inoculate 50 ml of LB medium. The culture was incubated at 37°C and 250 rpm (Aquatron, Infors AG, Switzerland) in a baffled flask until it reached an OD_{578} of 1.0. Cells were sedimented by centrifugation (2,600 x g, 15 min, 4°C) and suspended in 5 ml of SMMP. After adding 100 µl of freshly prepared sterile lysozyme solution (100 µg of lysozyme per ml SMMP), the protoplast

suspension was incubated at 37°C for 30 min with smooth shaking. Formation of protoplasts was monitored microscopically. After approximately 80 % of the rod shaped bacterial cells had formed coccoid protoplasts, the protoplasts were harvested (1,300 x g, 10 min, RT). The supernatant was decanted carefully and the protoplasts were suspended in 5 ml of SMMP. After a second washing step, the protoplasts were suspended in 5 ml of SMMP solution and mixed with 750 µl of 87 % (w/v) glycerol. They were either used directly for transformation or were frozen and stored in aliquots of 500 µl at -80°C for a period of no longer than 2 months.

Before transformation, protoplasts were tested for viability. Therefore, a 500 µl aliquot of protoplast solution was mixed with 2.5 ml of CR5-top agar as described below and was spread onto a LB medium agar plate without antibiotics. After incubation overnight, a thick film of *B. megaterium* cells should be seen. For the transformation of protoplasts, up to 3 µg of dried plasmid DNA were dissolved in 10 µl of SMMP for 20 min at 37°C. 500 µl of protoplast suspension were mixed with the DNA and transferred into 1.5 ml of PEG-P solution. 5 ml of SMMP solution were added after incubation for a period of 2 min at RT and were gently mixed with the suspension. The protoplasts were sedimented by centrifugation (1,300 x g, 10 min, RT), carefully suspended in 500 µl of SMMP solution and incubated at 30°C for 45 min without shaking followed by 45 min of smooth shaking at 300 rpm (Thermomixer compact, Eppendorf, Germany). Regenerated protoplasts were mixed with 2.5 ml of pre-warmed (42°C) CR5-top agar and spread on a pre-heated LB medium agar plate containing the required antibiotics. The plates were incubated at 30°C for up to 24 h. Colonies arising after this period of incubation were streaked onto new LB medium agar plates containing the required antibiotics.

SMMP solution

Antibiotic Medium No. 3 (Difco)	1.75 % (w/v)
Malic Acid	20.0 mM
NaOH	40.0 mM
$MgCl_2$ x 6 H_2O	20.0 mM
Sucrose	500.0 mM

PEG-P solution

PEG 6000	40.0 % (w/v)
Malic Acid	20.0 mM
NaOH	40.0 mM
$MgCl_2$ x 6 H_2O	20.0 mM
Sucrose	500.0 mM

CR5-top agar (2.5 ml)

Solution A	1.25 ml
Solution B	713.0 µl
8 x CR5 Salts	288.0 µl
L-Proline (12 % (w/v))	125.0 µl
D-Glucose (20 % (w/v))	125.0 µl

Solution A

Sucrose	602.0 mM
MOPS	58.0 mM
NaOH	30.0 mM

The pH was adjusted to 7.3 using NaOH. Sterilisation was performed using a sterile filter (0.2 µm pore size).

Solution B

Agar-Agar	4.0 % (w/v)
Casamino Acids	0.2 % (w/v)
Yeast Extract	10.0 % (w/v)

CR5-Salts

K_2SO_4	11.0 mM
$MgCl_2$ x 6 H_2O	394.0 mM
KH_2PO_4	3.0 mM
$CaCl_2$	159.0 mM

2.4.3 Preparation and Transformation of Naturally Competent *Bacillus megaterium*

The *B. megaterium* DSM319 plasmid strain pDFcomK$_{Bmeg}$ was cultivated for approximately 14 h at 37°C in TY-medium supplemented with 10 µg ml^{-1} tetracyline. This culture was used to inoculate fresh minimal medium containing 2.5 µg ml^{-1} tetracycline to a final OD_{600} of 0.15. Cultivation was proceeded at 37°C in baffled flasks at 250 rpm (Aquatron, Infors AG, Switzerland). After reaching an OD_{600} of 0.75, xylose was added to a final concentration of 0.5 % (w/v), 1.0 % (w/v), 1.5 % (w/v) and 2.0 % (w/v), respectively. An additional reference culture was left untreated. After another 3.5 h, 150 µl aliquots were taken and either supplemented with plasmid pSKE194 (200-600 ng) or with genomic DNA of *B. megaterium* YYBm1 (1-4 µg). After incubation at 30°C for 30 min, 300 µl of TY medium were added. The samples were further incubated at 30°C for 60 min and subsequently plated onto LB medium agar plates containing 2 µg ml^{-1} erythromycin. Colonies arsing after incubation at 30°C were analysed

microscopically and plated onto new agar plates containing 2 µg ml^{-1} erythromycin. The presence of the erythromycin resistance gene in transformants was proved by PCR using the oligonucleotides listed in Table 2.6.

Table 2.6: Oligonucleotides for the amplification of the erythromycin resistance gene.

Oligonucleotide Primer	Sequence (5'-3')
for Ery	GATAAGAATTGTTCAAAGC
rev Ery	TACTTCAAAACATAATATAG

Tryptone-Yeast Extract Broth (TY Medium)

Tryptone	1.0 % (w/v)
Yeast Extract	0.5 % (w/v)
NaCl	1.0 % (w/v)

Minimal Medium

Casamino Acids	0.02 % (w/v)
K_2HPO_4	62.0 mM
KH_2PO_4	44.0 mM
$(NH_4)_2SO_4$	15.0 mM
Na Citrate	6.5 mM
$MgSO_4$	800.0 µM
L-Tryptophane	100.0 µM
Fructose	27.8 mM

Fructose was sterilised by filtration (pore size 0.2 µm).

2.4.4 Preparation of Genomic DNA from *Bacillus megaterium*

The respective strains were grown in 50 ml LB medium to an OD_{600} of 3. The bacteria were harvested by centrifugation (4,000 x g, 10 min, 4°C) and suspended in 3.5 ml of lysis buffer supplemented with 0.2 mg/ml RNase A and 50 µg lysozyme. The samples were incubated at 37°C for 40 min. After addition of 500 µl N-laurosylsarcosinine (20 % (w/v)) and further incubation for 5 min at 37°C the suspension was mixed with one volume of phenol and incubated under constant motion for 25 min at room temperature (RT). After subsequent centrifugation (4,500 x g, 25 min, 4°C) the upper, aqueous phase was transferred into a fresh tube and submitted to 4 ml phenol/chloroform/isoamyl alcohol (24:24:1 (v/v/v)). Following 15 min of incubation, samples were centrifuged again (4,500 x g, 25 min, 4°C) and the phenol-chloroform extraction was repeated. The upper aqueous phase of this step was transferred into a fresh tube and DNA precipitation was initiated by the addition of 3 ml ice-cold isopropanol and 400 µl of a 3 M Na acetate solution (pH 4.8). The sample was

centrifuged (7,000 x g, 30 min, 4°C) and the DNA was washed twice with 70 % (v/v) ethanol. After evaporation of residual ethanol, the DNA was solubilised in 200 µl ddH_2O and stored at 4°C.

Lysis buffer

NaCl	100.0 mM
EDTA	50.0 mM

The pH was adjusted to 7.5 using acetic acid.

2.4.5 Preparation of Plasmid DNA from *Escherichia coli*

Plasmid DNA destined for protoplast transformation of *B. megaterium* was prepared from appropriate *E. coli* strains carrying the corresponding plasmid using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Alternatively, 4 ml of an overnight culture were harvested by centrifugation (18,000 x g, 2 min, RT) and the cells were suspended in 300 µl of buffer P1. 300 µl of buffer P2 were added, the sample was carefully mixed by inverting the tube and incubated at RT for 2 min. Next, 300 µl of buffer P3 were added, the sample was carefully mixed and further incubated on ice for 5 min. Following centrifugation (18,000 x g, 30 min, RT) 800 µl of the supernatant were added to 600 µl isopropanol in a fresh tube. Precipitation of plasmid DNA was allowed to proceed during a 10 min incubation step at 4°C, followed by a 30 min centrifugation step at RT (18,000 x g). The DNA was washed with 400 µl of 70 % (v/v) ethanol, centrifuged again (18,000 x g, 5 min, RT) and dried at 37°C. After all traces of ethanol had evaporated, the DNA was solubilised in 50 µl ddH_2O.

Buffer P1

Tris-HCl (pH 8.0)	50.0 mM
EDTA	10.0 mM
RNase A	100.0 µg ml^{-1}

Buffer P2

NaOH	200.0 mM
Na Dodecyl Sulphate	1.0 % (w/v)

Buffer P3

K Acetate	3.0 M

The pH was adjusted to 5.5 using acetic acid.

2.4.6 Preparation of Plasmid DNA from *Bacillus megaterium*

4 ml of an overnight culture were harvested by centrifugation (18,000 x g, 2 min, RT). The cells were suspended in 300 µl of TE(H)-buffer and incubated for 30 min at 37°C and 1,000 rpm (Thermomixer compact, Eppendorf). 300 µl of buffer P2 were added, the sample was carefully mixed by inverting the tube and incubated at RT for 2 min. Next, 300 µl of buffer P3 were added, the sample was again carefully mixed and further incubated on ice for 5 min. Following centrifugation (18,000 x g, 30 min, RT) 800 µl of the supernatant were added to 600 µl isopropanol in a fresh tube. Precipitation of plasmid DNA was allowed to proceed during a 10 min incubation step at 4°C, followed by a 30 min centrifugation step at RT (18,000 x g). The DNA was washed with 400 µl of 70 % (v/v) ethanol, centrifuged again (18,000 x g, 5 min, RT) and dried at 37°C. After all traces of ethanol had evaporated, the DNA was solubilized in 50 µl ddH_2O.

Buffer TE(H)

TRIS-HCl (pH 8.0)	50.0 mM
EDTA	10.0 mM
RNaseA	100.0 µg ml^{-1}
Lysozyme	30.0 µg ml^{-1}

Buffer P2

NaOH	200.0 mM
Na Dodecyl Sulphate	1.0 % (w/v)

Buffer P3

K Acetate	3.0 M

The pH was adjusted to 5.5 using acetic acid.

2.4.7 Determination of DNA Concentration

The concentration of plasmid DNA was determined by agarose gel electrophoresis. The plasmid was enzymatically linearised and visualised on an agarose gel (chapter 2.4.8). Using the software Quantity One (Bio-Rad, Munich, Germany), the concentration of the respective band in the agarose gel was determined in comparison to two bands of known concentrations.

The concentration and purity of genomic DNA was determined by measuring the absorbance at 260 nm and additionally at 280 nm to account for protein impurities. For a pure DNA solution an OD_{260} of 1 corresponds to a dsDNA concentration of 50 µg ml^{-1}.

The puritiy of the DNA solution was deduced from the ratio of OD_{260} to OD_{280}. DNA having an OD_{260}/OD_{280} ratio of 1.8 – 2.0 was considered as pure.

2.4.8 Electrophoretic Separation of DNA

For the analytical separation of DNA-fragments agarose gels (1 % (w/v) agarose in TAE-buffer) were prepared. DNA samples were mixed with DNA loading dye to facilitate loading and to indicate the progress of DNA migration. GeneRuler™ DNA Ladder Mix or MassRuler™ DNA Ladder Mix (Fermentas, St. Leon-Rot, Germany) were used as size standards according to the manufacturer's instructions. Depending on the size of the gel, a voltage of 80-100 V was applied. After electrophoresis, gels were incubated in an ethidium bromide solution for 30 min. The DNA was detected via its fluorescence under UV light (λ = 312 nm).

TAE-Buffer

TRIS-Acetate (pH 8.0)	40.0	mM
EDTA	1.0	mM

DNA Loading Dye

Bromphenol Blue	350.0	µM
Cylene Cyanol FF	450.0	µM
Glycerol	50.0	% (w/v)

Ethidium Bromide Solution

Ethidium Bromide	0.1	% (w/v)

2.4.9 Amplification of DNA Fragments by Polymerase Chain Reaction

For amplification of DNA by PCR, oligonucleotide primers for each DNA fragment of interest were designed. Recognition sequences for restriction endonucleases were inserted into these primers. All oligonucleotide primers are listed in Table 2.7. Primers were purchased from Metabion International AG (Martinsried, Germany) or biomers.net GmbH (Ulm, Germany).

Genomic DNA or plasmid DNA in appropriate concentrations were used as templates in individual PCRs. Phusion™ polymerase (Finnzymes; Espoo; Finland) was applied as the polymerase of first choice for the amplification of large DNA fragments (>1,000 bp). Taq DNA polymerase (BioTherm®, GeneCraft, Lüdinghausen, Germany) was used in

all other cases. Both polymerases were applied according to the manufacturer's instructions.

Table 2.7: Oligonucleotide primers. Nucleotides comprising restriction endonuclease recognition sequences are underlined.

Oligonucleotide Primer	Designated Use	Sequence (5'-3')
Codon Usage and Recombinant Protein Production in *Bacillus megaterium*		
gfp+arg (CGG) for	Generation of pMGBm63	GAATTACTAGTCGGCGGCGGCGGTCGAAGATC TGGGCTAGCAAAG
gfp rev		AATATCGGCCGTTATTTGTAGAGCTCATCC
gfp+arg (CGT) for	Generation of pMGBm64	GAATTACTAGTCGTCGTCGTCGTTCGAAGATCT GGGCTAGCAAAG
gfp rev		AATATCGGCCGTTATTTGTAGAGCTCATCC
gfp+leu (CTC) for	Generation of pMGBm65	GAATTACTAGTCTCCTCCTCCTCTCGAAGATCT GGGCTAGCAAAG
gfp rev		AATATCGGCCGTTATTTGTAGAGCTCATCC
gfp+leu (TTA) for	Generation of pMGBm66	GAATTACTAGTTTATTATTATTATCGAAGATCTG GGCTAGCAAAG
gfp rev		AATATCGGCCGTTATTTGTAGAGCTCATCC
gfp+ser (TCA) for	Generation of pMGBm67	GAATTACTAGTTCATCATCATCATCGAAGATCTG GGCTAGCAAAG
gfp rev		AATATCGGCCGTTATTTGTAGAGCTCATCC
gfp+ser (TCC) for	Generation of pMGBm68	GAATTACTAGTTCCTCCTCCTCCTCGAAGATCT GGGCTAGCAAAG
gfp rev		AATATCGGCCGTTATTTGTAGAGCTCATCC
gfp+gly (GGG) for	Generation of pMGBm70	GAATTACTAGTGGGGGGGGGGGGTCGAAGATC TGGGCTAGCAAAG
gfp rev		AATATCGGCCGTTATTTGTAGAGCTCATCC
gfp+gly (GGA) for	Generation of pMGBm71	GAATTACTAGTGGAGGAGGAGGATCGAAGATCT GGGCTAGCAAAG
gfp rev		AATATCGGCCGTTATTTGTAGAGCTCATCC
gfp+ala (GCC) for	Generation of pMGBm72	GAATTACTAGTGCCGCCGCCGCCTCGAAGATCT GGGCTAGCAAAG
gfp rev		AATATCGGCCGTTATTTGTAGAGCTCATCC
gfp+ala (GCT) for	Generation of pMGBm73	GAATTACTAGTGCTGCTGCTGCTTCGAAGATCT GGGCTAGCAAAG
gfp rev		AATATCGGCCGTTATTTGTAGAGCTCATCC
gfp+arg (AGG) for	Generation of pMGBm78	GAATTACTAGTAGGAGGAGGAGGTCGAAGATCT GGGCTAGCAAAG
gfp rev		AATATCGGCCGTTATTTGTAGAGCTCATCC
gfp+thr (ACC) for	Generation of pMGBm96	GAATTACTAGTACCACCACCACCTCGAAGATCT GGGCTAGCAAAG
gfp rev		AATATCGGCCGTTATTTGTAGAGCTCATCC
gfp+thr (ACA) for	Generation of pMGBm97	GAATTACTAGTACAACAACAACATCGAAGATCT GGGCTAGCAAAG
gfp rev		AATATCGGCCGTTATTTGTAGAGCTCATCC

Table 2.7 (continued): Oligonucleotide primers.

Oligonucleotide Primer	Designated Use	Sequence (5'-3')
for P_{spac}	Generation of pYZ5-P_{spac}	GTAATGAGCTCTTCAGAACGCTCGGTTGCCG
rev P_{spac}		GTAATCCCGGGTTGCTAGCCGGCCGATCGATAGCGCTGGTACCC
for PthrGGU (SacI)	Generation of ptRNA $^{Thr}_{GGU}$	GTAATGAGCTCCTATTTTCTTCCCTTCTTTTG
rev thrGGU (XmaI)		GAATTCCCGGGTAAAGTCAACACAAATAAAAAAACC
T7 RNA Polymerase Dependent Gene Expression in *Bacillus megaterium*		
XylR_for_YZ5	Generation of pMGBm19	GATATGGCGCCCTTTGCGTTCACTTAACTAACTTATAGG
MCS_rev_YZ5		CATATGGATCCGTTTGCGCATTCACAGTTCTCC
RNAP_for	Generation of pT7-RNAP	GAATTACTAGTGGAGGAGGAGGAATGAACACGATTAACATCG
RNAP_rev		CTATACCCGGGTTACGCGAACGCGAAGTC
GFP_for	Generation of pP_{T7}-GFP	GAATTACTAGTTCGAAGATCTGGGC
GFP_rev		CTATAGCATGCGGCCGCGAATTC
LEV_for	Generation of pP_{T7}-Lev	GGAAATGTACAATGAAAAAAG
LEV_rev		CTATAGAGCTCTTAGTGATGGTGATGGTG
***Bacillus megaterium* – an Alternative Host for the Production of Recombinant Proteins of Eukaryotic Origin**		
for2 kbd b (SacI/NarI)	Generation of pP_{xylA}-kbdb-his_6	GAATTGAGCTCAAGGCGCCGCTGAATGTGAATTAGAACCACACAC
rev2 kbd b (SphI)		CGAATGCATGCTGGTGGTGATGGTGGAGCTTCTTTTAATGGTAATAAAC
for1 kbd b (SacI/NarI)	Generation of pP_{xylA}-his_6-kbdb	GAATTGAGCTCAAGGCGCCCGTGGTTCACACCACC
rev1 kbd b (SphI)		CACGTGCATGCTTATTCTTTTAATGGTAATAAAC
for *dewA* (*Sac*I/*Nar*I):	Generation of pP_{xylA}-sp_{lipA}-dewA-his_6	GAATTGAGCTCAAGGCGCCGCTGAATTACCAGCTTCAGCTGC
rev *dewA* (*Sph*I)		CACGTGCATGCTTATTAGTGATGGTGGTGG
Development of Natural Competence in *Bacillus megaterium*		
for pDFBm6 (BglII)	Generation of pDFHis-$comK_{Bmeg}$	CTATAAGATCTCTATGACAAATGATGAAAAG
rev pDFBm6 (EagI)		CTATACGGCCGTTATGCCAAGACAACCGGAC
for pDFBm7 (BsrGI)	Generation of pDFcomK$_{Bmeg}$	CTATATGTACAATGACAAATGATGAAAAG
rev pDFBm7 (EagI)		CTATACGGCCGTTATGCCAAGACAACCGGAC
for pDFBm8 (KpnI)	Generation of pDFgfp	CTATAGGTACCATGGTCCAAACTAGTTCGAAG
rev pDFBm8 (EcoRI)		CTATAGAATTCATTATTTGTAGAGC

Table 2.7 (continued): Oligonucleotide primers.

Oligonucleotide Primer	Designated Use	Sequence (5'-3')
for pDFBm11 (XbaI_AgeI)	Generation of pDFrecA-gfp	CTATATCTAGACCGGTCTTGATGCGCAAACCAATCC
rev pDFBm11 (KpnI)		CTATAGGTACCTCCTCCTCTATCTCTTTGAAATC
for pDFBm12 (AgeI)	Generation of pDFcomG-gfp	CTATAACCGGTCTATCATCGTATACCGCTAG
rev pDFBm12 (KpnI)		CTATAGGTACCATCACTCCTTTATTTGTAATC
for pDFBm14 (BsrGI)	Generation of pDFcomK_{Bsu}	CTATATGTACAATGAGTCAGAAAACAGACGC
rev pDFBm14 (SphI)		CTATAGCATGCCTAATACCGTTCCCCGAGCTC

2.4.10 Phosphorylation and Hybridisation of Oligonucleotides

300 pmol of oligonucleotides were phosphorylated using T4 polynucleotide kinase according to the manufacturer's instructions. After heat inactivation of the enzyme at 65°C for 20 min the two corresponding phosphorylated oligonucleotides were mixed and incubated at 95°C for 3 min. Annealing was allowed to proceed at the calculated T_m for 1 min. After subsequent incubation at 55°C for 1 min the hybridised oligonucleotides were used for ligation reactions.

Oligonucleotides used in the study are listed in Table 2.8.

Table 2.8: Oligonucleotides. Nucleotides comprising restriction endonuclease recognition sequences after hybridisation of corresponding oligonucleotides are underlined.

Oligonucleotide Primer	Designated Use	Sequence (5'-3')
T7 RNA Polymerase Dependent Gene Expression in *Bacillus megaterium*		
Pϕ10_for	Generation of pStop1622-Pϕ10	TTAAGGCGAAATTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCGAATATTAATTAACCAAGGAGGTGAAAT
Pϕ10_rev		GTACATTTCACCTCCTTGGTTAATTAATATTCGGGAAACCGTTGTGGTCTCCCTATAGTGAGTCGTATTAATTTCGCC
Tϕ10_for	Generation of pP_{T7}	CCGGCTAATCGCGACCGGTTAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTATAT
Tϕ10_rev		CCGGATATAGTTCCTCCTTTCAGCAAAAAACCCCTCAAGACCCGTTTAGAGGCCCCAAGGGGTTATGCTAGTTAACCGGTCGCGATTAGCCGGCATG

Table 2.8 (continued): Oligonucleotides.

Oligonucleotide Primer	Designated Use	Sequence (5'-3')
***Bacillus megaterium* – an Alternative Host for the Production of Recombinant Proteins of Eukaryotic Origin**		
Sense New His	Generation of pNewHis1525	CGCTCACCACCATCACCACCACTAA
Anti-Sense New His		CCGGTTAGTGGTGGTGATGGTGGTGAGCGCATG

2.4.11 Enzymatic Digestion of DNA

Digestion of double stranded DNA (vectors and PCR products) was carried out using restriction endonucleases. Reaction buffers, concentrations of enzymes and DNA concentrations as well as incubation temperatures were chosen according to manufacturer's instructions. The restriction was allowed to proceed for 3 h or overnight at 37°C. Restriction endonucleases in the entire sample were removed by gel electrophoresis (chapter 2.4.8). The DNA was visualised using the GelStar Nucleic Acid Gel Stain (Lonza, Basel, Switzerland) on a blue light detector (Flu-O-blu, Biozym, Germany) and a yellow filter. The DNA fragment was excised from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions.

2.4.12 Ligation of DNA Fragments

25 - 200 ng of plasmid DNA were used. Insert-DNA was added in excess (insert to vector ratio with regard to molar concentrations of 2:1 to 10:1) to a final volume of 20 µl. Ligation of vector- and insert-DNA was carried out according to the manufacturer's instructions using T4 Ligase. Additionally, controls without insert and without ligase were carried out. All reactions were incubated for 20 min at 25°C or at 17°C overnight.

2.4.13 DNA Sequencing

The successful modification of DNA was confirmed by sequence determination of the respective DNA region based on the principle of the Sanger dideoxy-method (Sanger *et al.* 1977). The sequencing reactions were conducted either on-site with an ABI PRISM 310 Genetic Analyser (Applied Biosystems, Perkin Elmer, Boston, USA) or by GATC Biotech AG (Konstanz, Germany). If sequencing was performed on-site the required preparatory PCR with fluorescence-labelled ddNTPs and the purification of the PCR product were carried out as described by the manufacturer. The analysis of all

sequencing results was done using the computer software Sequence Analysis v5.2 (Applied Biosystems, Perkin Elmer, Boston, USA), Chromas (Technelysium, Austria) and Vector NTI (Invitrogen, Karlsruhe, Germany).

2.4.14 Preparation of RNA for Northern Blot Analysis

For preparations of RNA, 25 ml of cell culture were added to 25 ml of ice-cold killing buffer. The cells were harvested by centrifugation (7,155 x g, 5 min, 4°C). The sedimented bacteria were suspended in 200 µl of supernatant, immediately dropped into a Teflon disruption vessel, filled, and precooled with liquid N_2. The cells were disrupted with a Mikro-Dismembrator S instrument (B. Braun Biotech International, Melsungen, Germany) for 2 min at 2,600 rpm. The resulting frozen powder was suspended in 1 ml of pre-warmed (50°C) cell lysis solution. After complete cell lysis, the solution was immediately placed on ice. The RNA was extracted twice with acidic phenol-chloroform-isoamyl alcohol (25:24:1 (v/v/v)) and once with chloroform-isoamyl alcohol (24:1 (v/v)). After ethanol precipitation, the RNA was resuspended in 180 µl of RNA storage buffer and 20 µl of DNase buffer containing 15 U of DNase I and then was incubated for 30 min at room temperature. 20 µl of 250 mM EDTA (pH 7.0) were added, followed by phenol-chloroform extraction and ethanol precipitation. Subsequently, the RNA was dissolved in 50 µl of ddH_2O.

Killing Buffer

Tris-HCl (pH 7.5)	20.0	mM
$MgCl_2$	5.0	mM
NaN_3	20.0	mM

Cell Lysis Solution

Na Acetate (pH 5.2)	4.0	M
Guanidine Thiocyanate	25.0	mM
N-Laurylsarcosine	0.5	% (w/v)

RNA Storage Buffer

$NaPO_4$ (pH 4.5)	20.0	mM
EDTA	1.0	mM

Dnase Buffer

Na Acetate (pH 4.5)	200.0	mM
$MgCl_2$	180.0	mM
NaCl	100.0	mM

2.4.15 Northern Blotting and Detection of Specific Transcripts

10 µg of RNA was separated under denaturing conditions in a 1% agarose-670 mM formaldehyde-MOPS gel, stained with ethidium bromide, and transferred to a positively charged nylon membrane (Roche Diagnostics, Mannheim, Germany) by vacuum blotting. The RNA was crosslinked to the membrane by UV-light. The approximate sizes of the mRNAs were estimated by the use of RNA standards (Bethesda Research Laboratories, Bethesda, USA) labeled with digoxigenin.

A digoxigenin-labeled RNA probe was synthesised *in vitro* with T7 RNA polymerase (DIG RNA Labeling Kit, Roche Diagnostics, Mannheim, Germany) and a 724 bp *kbd b*-specific PCR fragment as a template, previously amplified from pP_{xylA}-his_6-kbdb using the oligonucleotide primers listed in Table 2.9. The labeling reaction was performed according to the manufacturer's instructions. The RNA probe was precipitated by the addition of 0.1 volumes of 4 M LiCl and 3 volumes of -20°C cold pure ethanol. After storage overnight at -80°C, the sample was centrifuged (14,000 x g, 15 min, RT), the supernatant was removed and the precipitated RNA probe was washed once with 100 µl of -20°C pure ethanol. The supernatant was removed after an additional centrifugation step (14,000 x g, 5 min, RT), the dried digoxigenin-labeled RNA probe was suspended in 20 µl ddH_2O and finally stored at -80°C.

Table 2.9: Oligonucleotide primers for the generation of a *kbd b*-specific RNA probe.

Oligonucleotide Primer	Sequence (5'-3')
for KBD B	CCACGGTTCAGCTTGTGAAT
rev KBD B	CTAATACGACTCACTATAGGGAGATGGATCGAAGAAACCTTTTG

Prehybridisation of the membrane was performed in hybridisation solution for 1 h at 50°C. Hybridisation was carried out in 20 ml hybridisation solution supplemented with 9 µg of digoxigenin-labelled RNA probe at 50°C overnight. After hybridisation the membrane was washed at 50°C three times with wash buffer for 20 min each. Subsequently, the membrane was incubated in maleic acid buffer for 5 min at RT and further on in blocking buffer for 1 h at RT. Anti-digoxigenin Fab fragments conjugated with alkaline phosphatase were diluted in blocking buffer (1:13,300) and applied to the membrane. After 30 min of incubation the membrane was washed at RT four times with maleic acid buffer for 10 min each and finally with substrate buffer for 5 min. CDP-*Star* solution (Roche Diagnostics, Mannheim, Germany) was diluted 100-fold in substrate buffer and applied to the membrane. The documentation of chemiluminescent signals was carried out by sealing the wet membrane in a plastic bag and placing it in close contact to an X-ray film.

Blocking Stock Solution

Blocking Reagent	10.0	% (w/v)
Maleic Acid	100.0	mM
NaCl	150.0	mM
NaOH	0.7	% (w/v)
Tween 20	0.3	% (v/v)

The pH was adjusted to 7.5 using NaOH. Tween 20 was added after heat sterilisation.

Prehybridisation Solution

Na_2HPO_4 x 7 H_2O	250.0	mM
EDTA	1.0	mM
SDS	20.0	% (w/v)

The pH was adjusted to 7.2 using phosphoric acid.

Hybridisation Solution

Blocking Stock Solution	5.0	% (w/v)
Prehybridisation Solution	95.0	% (w/v)

Wash Buffer

Na_2HPO_4 x 7 H_2O	20.0	mM
EDTA	1.0	mM
SDS	1.0	% (w/v)

The pH was adjusted to 7.2 using phosphoric acid.

Maleic Acid Buffer

Maleic Acid	100.0	mM
NaCl	3.0	M
NaOH	0.5	% (w/v)
Tween 20	0.3	% (v/v)

The pH was adjusted to 8.0 using NaOH. Tween 20 was added after heat sterilisation.

Blocking Buffer

Blocking Stock Solution	5.0	(w/v)
Maleic Acid Buffer	95.0	(w/v)

2.5 Protein Biochemical Techniques

2.5.1 Expression of Recombinant Genes

The respective *B. megaterium* plasmid strains were streaked onto LB medium agar plates containing the appropriate antibiotics and cultivated for approximately 14 h at 37°C. Single colonies were used to inoculate 50 ml LB medium supplemented with

appropriate antibiotics. The cultivation was performed at 37°C for approximately 14 h and gentle shaking at 100 rpm (Aquatron, Infors AG, Switzerland) in baffled flasks. An aliquot of 1 ml was used to inoculate 100 ml of respective growth medium (LB-, TB- and A5+4 medium, respectively) supplemented with appropriate antibiotics. In case of using minimal medium the main cultures were inoculated with the pre-cultures to a final OD_{600} of 0.15. Cultivations were performed in baffled flasks at 37°C and shaking at 250 rpm (Aquatron, Infors AG, Switzerland). Cultivations in 96-well plates were performed at 37°C and 1,000 rpm (Thermo Shaker PST-60HL-4, Lab4you). After reaching an OD_{578} of 0.4, recombinant gene expression was induced by the addition of 0.5 % (w/v) xylose by default. Recombinant gene expression of cultivations in minimal medium was induced by the addition of varying concentrations of xylose (0.5 - 3.0 % (w/v)) after reaching an OD_{600} of 0.6.

2.5.2 Preparation of Intracellular Protein Fractions

Approximately 3 x 10^9 *B. megaterium* cells were harvested by centrifugation (18,000 x g, 10 min, 4°C). The supernatant was removed completely and the cells were suspended in 30 µl of lysis buffer. After subsequent incubation at 37°C for 30 min and vigorous shaking at 1,000 rpm (Thermomixer compact, Eppendorf, Germany) the samples were centrifuged (18,000 x g, 30 min, 4°C). 26 µl of the supernatant containing the soluble proteins were mixed with 13 µl of SDS loading dye. The sediment, containing cell debris and insoluble proteins, was suspended in 30 µl of urea buffer and centrifuged again (18,000 x g; 30 min; 4°C). 26 µl of the supernatant were mixed with 13 µl of SDS loading dye. 7.5 µl of the prepared samples which corresponded to proteins equivalent to 0.5 x 10^9 cells were analysed by SDS-PAGE (chapter 2.5.4).

Lysis Buffer

Na_3PO_4	100.0	mM
$MgSO_4$	10.0	mM
Lysozyme	5	mM
Benzonase	50	U ml^{-1}

The pH was adjusted to 5.5 using H_3PO_4. Benzonase (Merck KGaA, Darmstadt, Germany) was added prior to use.

Urea Buffer

Tris-HCl (pH 7.5)	50.0	mM
Urea	8.0	M

SDS Loading Dye

Tris-HCl (pH 6.8)	100.0 mM
Glycerin	40.0 % (w/v)
β–Mercaptoethanol	2.0 mM
SDS	110.0 mM
Bromphenol Blue	3.0 mM

2.5.3 Preparation of Extracellular Protein Fractions

Extracellular proteins were precipitated by ammonium sulphate. For this purpose, cell-free samles were obtained by centrifugation (2,600 x g, 15 min, 4°C). 1.5 ml of the supernatant were supplemented with ammonium sulphate to a final concentration of either 15 % (w/v) or 44 % (w/v). If using A5+4 medium for cultivation trytone (15 mg per 1.5 ml of cell-free culture supernatant) was added in addition in order to facilitate the precipitation of extracellular proteins. The samples were incubated at gentle shaking at 4°C for 2 h. Precipitated proteins were collected by centrifugation (18,000 x g, 30 min, 4°C). After removal of the supernatant, the proteins were suspended in 10 µl of urea buffer and 5 µl of SDS loading dye and analysed by SDS-PAGE (chapter 2.5.4).

Urea Buffer

Tris-HCl (pH 7.5)	50.0 mM
Urea	8.0 M

SDS Loading Dye

Tris-HCl (pH 6.8)	100.0 mM
β–Mercaptoethanol	2.0 mM
SDS	110.0 mM
Bromphenol Blue	3.0 mM
Glycerin	40.0 % (w/v)

2.5.4 Electrophoretic Separation of Proteins (SDS-PAGE)

Proteins were analysed by SDS-PAGE as described by Laemmli (Lämmli 1970) with modifications by Righetti (Righetti *et al.* 1990) for discontinuous SDS-PAGE. Protein samples were denatured by heating to 95°C for 5 min in SDS loading dye. Samples were loaded onto the gel which was run at 40 mA. During electrophoresis, proteins were first focussed in the stacking gel and subsequently separated according to their relative molecular mass in the running gel. The size standards employed were Protein Molecular Weight Marker (Fermentas, St. Leon-Rot, Germany) and PageRuler

Prestained Protein Ladder (Fermentas, St. Leon-Rot, Germany). Gels were stained with staining solution and destained with destaining solution until the protein bands were clearly visible.

Acrylamide Stock Solution	
Acrylamide	30.0 % (w/v)
TEMED	1.0 % (w/v)

Ammonium Peroxodisulphate Solution	
Ammonium Peroxodisulphate	10.0 % (w/v)

Stacking Gel (6 % (v/v), Sufficient for 4 Mini-Gels	
dH_2O	5.5 ml
500 mM Tris-HCl (pH 6.8); 0.4 % (w/v) SDS	2.5 ml
Acrylamide Stock Solution	2.0 ml
TEMED	10.0 µl
Ammonium Peroxodisulphate Solution	100.0 µl

Running Gel (12 % (v/v), Sufficient for 4 Mini-Gels	
dH_2O	7.0 ml
1.5 M Tris-HCl (pH 8.8); 0.4 % (w/v) SDS	5.0 ml
Acrylamide Stock Solution	8.0 ml
TEMED	20.0 µl
Ammonium Peroxodisulphate Solution	200.0 µl

Running Gel (15 % (v/v), Sufficient for 4 Mini-Gels	
dH_2O	5.0 ml
1.5 M Tris-HCl (pH 8.8); 0.4 % (w/v) SDS	5.0 ml
Acrylamide Stock Solution	10.0 ml
TEMED	20.0 µl
Ammonium Peroxodisulphate Solution	200.0 µl

SDS Loading Dye	
Tris-HCl (pH 6.8)	100.0 mM
β–Mercaptoethanol	2.0 mM
SDS	110.0 mM
Bromphenol Blue	3.0 mM
Glycerin	40.0 % (w/v)

Electrophoresis Buffer	
Tris-HCl (pH 8.8)	50.0 mM
Glycine	385.0 mM
SDS	0.1 % (w/v)

Staining Solution	
Ethanol	30.0 % (v/v)
Acetic Acid	10.0 % (v/v)
Coomassie Brilliant Blue G-250	0.25 % (w/v)

Destaining Solution	
Ethanol	30.0 % (v/v)
Acetic Acid	10.0 % (v/v)

2.5.5 Western Blotting and Immunological Detection of Immobilised Proteins

For further analysis, proteins separated by SDS-PAGE were transferred in a semi-dry process onto a polyvinylidenfluorid (PVDF)-membrane (Roth, Karlsruhe, Germany). Prior to blotting, the PVDF-membrane was incubated in methanol for 10 min. Afterwards, the PVDF-membrane as well as the SDS-gel and the blotting paper was incubated in transfer-buffer and assembled in the following order on the blotting apparatus: Cathode, blotting paper, PVDF-membrane, SDS-gel, blotting paper, anode. A current of 0.8 mA/cm^2 was applied for 15 min at 15 V. Non-specific binding sites of the membrane were saturated overnight in blocking solution at 4°C and gentle shaking. Proteins immobilised on a PVDF-membrane were specifically detected by antibodies. Primary antibodies were directed against T7 RNAP, KBD B and His_6-tag, respectively. Secondary antibodies were directed against the primary antibody and were either coupled to an alkaline phosphatase or to the horseradish peroxidase. Incubation with the primary antibody was carried out in blocking solution for 1 h (RT) and gentle shaking. The membrane was washed three times for 10 min with washing solution and incubated for 45 min with the secondary antibody. Afterwards, the membrane was washed four times for 10 min with PBS/Tween buffer.

In case of using a secondary antibody coupled to an alkaline phosphatase, the membrane was incubated for 5 min in alkaline phosphatase buffer and subsequently in alkaline phosphatase staining solution until signals became visible.

In case of using a secondary antibody coupled to the horesradish peroxidase, the membrane was incubated for 5 min in working solution consisting of equal volumina of

Luminol/Enhancer Solution and Stable Peroxidase Solution (SuperSignal West Pico Mouse IgG Detection Kit, Thermo Fisher Scientific, Rockford, USA). Afterwards, an X-ray film was exposed to the membrane and was processed. The exposure time varied between 1-2 min depending on the intensity of the signal.

Towbin Buffer	
Tris-HCl (pH 8.5)	25.0 mM
Glycine	150.0 mM

10x PBS	
NaCl	1.37 M
KCl	27.0 mM
Na_2HPO_4	50.0 mM
K_2HPO_4	15.0 mM

PBS/Tween Buffer	
10x PBS	10.0 % (v/v)
Tween 20	0.1 % (v/v)

Blocking Solution	
Skim Milk Powder	5.0 % (w/v)
In PBS/Tween Buffer.	

Washing Solution	
Skim Milk Powder	0.5 % (w/v)
In PBS/Tween Buffer.	

Alkaline Phosphatase Buffer	
Tris-HCl (pH 9.5)	100.0 mM
NaCl	100.0 mM
$MgCl_2$	5.0 mM

Alkaline Phosphatase Staining Solution	
Alkaline Phosphatase Buffer	10.0 ml
NBT-Solution (50 mg ml^{-1} in 70 % DMF)	66.0 µl
BCIP-Solution (50 mg ml^{-1} in DMF)	33.0 µl

Primary Antibodies	**Dilution**
mc-Rabbit Anti KBD B (BASF SE, Ludwigshafen, Germany)	1 : 500,000
mc-Mouse Anti T7 RNAP (Novagen, Darmstadt, Germany)	1 : 10,000
mc-Mouse Anti-His_6 (GE Healthcare, Uppsala, Sweden)	1 : 2,000

Secondary Antibodies	Dilution
pc-Goat α Mouse IgG-Fc-Alkaline Phosphatase (Sigma-Aldrich, St. Louis, USA)	1 : 5,000
pc-Goat α Rabbit IgG (H+L)-Alkaline Phosphatase (Thermo Fisher Scientific, Rockford, USA)	1 : 15,000
Goat α Mouse IgG (H+L)-Horseradish Peroxidase (Thermo Fisher Scientific, Rockford, USA)	1 : 10,000

2.5.6 Purification of Extracellular His_6-Tagged Fusion Proteins

1 ml of Chelating Sepharose FF (GE Healthcare; Uppsala; Sweden) was incubated with 1 ml of 100 mM $NiSO_4$ for approximately 10 min at RT. Afterwards, the affinity material was washed with 5 ml of washing buffer and added to 90 ml of cell-free culture supernatant. After 30 min of incubation at RT and gentle shaking the affinity material was sedimented by centrifugation (2,600 x g, 2 min, 4°C) and washed twice with 1.5 ml of washing buffer. Proteins were step-eluted using increasing concentrations of imidazole in washing buffer (200 mM and 500 mM, respectively).

Washing Buffer	
Tris-HCl (pH 8.0)	50.0 mM

2.5.7 Fluorescence Measurements of Green Fluorescent Protein

GFP production of cultivations in shaking flasks was detected by performing fluorescence measurements (Luminescence Spectrometer LS50B, PerkinElmer, Boston, USA). Samples were excited at 475 nm and fluorescence emission was detected at 512 nm. For the determination of GFP production over the time course of cultivation, cells were harvested at different times after induction of gene expression by centrifugation (14,000 x g, 10 min, 4°C) and were suspended appropriately in Na phosphate buffer (100 mM, pH 7.0). GFP quantities were calculated using the following equation (Biedendieck *et al.* 2007c):

$$GFP\ [mg\ ml^{-1}] = \text{relative emission maxima} \times 3.42 \times 10^{-6} \times \text{dilution factor}$$

Relative GFP production yields of cultivations in 96-well plates were obtained using an microplate reader (Fusion, Perkin Elmer, Massachusetts, USA) with an excitation wave length of 485 nm. The emission was recorded at 530 nm.

2.5.8 Determination of Levansucrase Activity

The levansucrase enzyme activity of various preparations described in this investigation was measured via the release of fructose and glucose from sucrose. The total amount of fructose and glucose was determined using dinitrosalicylic acid as described in the D.N.S. method (Sumer and Howell 1935). Using this method, the observed rate of total fructose and glucose formation is the sum of the rate of released glucose (v_G) and the rate of released fructose (v_F).

The levansucrase LevΔ773 from *Lactobacillus reuteri*, previously described by Van Hijum *et al.* (2004) was used as model. Van Hijum *et al.* (2004) reported that the recombinant levansucrase LevΔ773MycHis purified from *E. coli* and incubated with sucrose follows Michaelis-Menten kinetics. For the release of glucose (G) and fructose (F) a K_M^G of 9.7 mM, a k_{cat}^G of 147 s^{-1}, a K_M^F of 11.3 mM and a k_{cat}^F of 117 s^{-1} were determined. In the steady state, the substrate concentration [S] was assumed to be [S_0], which was found about 100 mM sucrose. From the rate of released reducing sugars ($v_G + v_F$), the rate of glucose (v_G) release was calculated using the following formula:

$$\frac{v_G}{v_G + v_F} = \frac{\frac{V_{\max}^G \cdot [S]}{K_M^G + [S]}}{\frac{V_{\max}^G \cdot [S]}{K_M^G + [S]} + \frac{V_{\max}^F \cdot [S]}{K_M^F + [S]}} = \frac{\frac{k_{cat}^G \cdot [S]}{K_M^G + [S]}}{\frac{k_{cat}^G \cdot [S]}{K_M^G + [S]} + \frac{k_{cat}^F \cdot [S]}{K_M^F + [S]}} = 0.560$$

All D.N.S. assays were performed at 37°C to avoid abiotic hydrolysis of sucrose (Ozimek *et al.* 2004). Levansucrase containing solutions were incubated in reaction buffer (van Hijum *et al.* 2004) in a total volume of 1.5 ml and at 1,000 rpm (Thermomixer compact, Eppendorf, Germany). Growth medium containing levansucrase was tested after 50-fold dilution. Samples were taken at different times after the reaction was started. To stop the reaction, samples were diluted equally with D.N.S. reagent and stored in the dark. In parallel, standard solutions containing 0.0 - 2.0 mg l^{-1} glucose were mixed equally with D.N.S. reagent. Samples and standards were boiled for 5 min at 100°C and immediately cooled down on ice. For quantification, samples and standards were diluted 1:6 with H_2O and the OD was measured at 540 nm.

Reaction Buffer (pH 5.4)	
$Na(C_2H_3O_2)$	25.0 mM
Sucrose	100.0 mM
$CaCl_2$	1.0 mM
NaN_3	0.7 µM

D.N.S. Reagent	
3,5-Dinitrosalicylic Acid	43.8 mM
$KNa_4C_4H_4O_6$	1.074 M
NaOH	400.0 mM

2.5.9 Coating of Sufaces with Hydrophobin

Glas was used as a representative for hydrophilic surfaces and Teflon® for hydrophobic surfaces. The materials were incubated overnight at 80°C in coating buffer with 50 µg ml^{-1} hydrophobin. After washing with dH_2O the coated materials were incubated for 10 min in 1 % (w/v) SDS-solution at 80°C and washed again with dH_2O. The surfaces were dryed at room temperature and the contact angle of a water droplet with a volume of 5 µl on hydrophobin-coated surfaces was determined (Dataphysics Contact Angle System OCA 15+, DataPhysics Instruments GmbH, Filderstadt, Germany). Contact angles of water droplets on uncoated surfaces were taken as control.

Coating Buffer	
Tris-HCl (pH 8.0)	50.0 mM
$CaCl_2$	1.0 mM

2.6 Analytical Techniques

2.6.1 Flow Cytometric Analyses

Flow cytometric measurements were performed in order to detect morphological changes within a bacterial population. For this purpose cells were harvested by centrifugation (14,000 x g, 2 min, RT) and suspended in fresh minimal medium to a final concentration of approximately 0.5 - 5 x 10^8 cells per ml. 25,000 events were analysed per sample using the flow cytometer Cytometrics FC 500 MPL (Beckmann Coulter GmbH, Krefeld, Germany). The intensity of forward light scattering was taken as a measure for the relative size of individual particles.

2.6.2 Evaluation of ComK-Induced Lysin Production

The *B. megaterium* plasmid strains DSM319 carrying pMM1520 and DSM319 carrying pDFcomK$_{Bmeg}$ were cultivated in parallel in LB medium at 37°C in baffled flasks at 250 rpm (Aquatron, Infors AG, Switzerland). After reaching an OD_{600} of 0.4, the cultures were supplemented with 0.5 % (w/v) xylose and further cultivated for 7 h. The bacteria were sedimented by centrifugation (2,600 x g, 15 min, 4°C) and the supernatants were filtered through a sterile filter (pore width 0.2 µm). These cell-free culture supernatants were subsequently added each to a final volume of 50 % (v/v) to an exponentially growing culture of *B. megaterium* DSM319 in LB medium. Cultivations were performed for 18 h at 37°C and 250 rpm (Aquatron, Infors AG, Switzerland). The OD_{600} was measured as well as microscopic images were taken in continuous intervals.

2.6.3 Fluorescence Microscopy

Fluorescence microscopy was performed with individual *B. megaterium* plasmid strains. Cells were diluted appropriately in minimal medium and analysed microscopically (Axiovert 200M, Carl Zeiss AG, Jena, Germany). Each image section was documented under phase contrast and fluorescence excitation using appropriate filters for the detection of GFP.

3 RESULTS AND DISCUSSION

3.1 T7 RNA Polymerase Dependent Gene Expression in *Bacillus megaterium*

The choice of promoter has significant influence on product yields in recombinant gene expression. Typically, promoters are recognized by the host RNA polymerase which then in turn transcribes the gene of interest. However, some very efficient bacterial expression systems rely on the activity of bacteriophage originated DNA dependent RNA polymerases. The most prominent of those expression systems was already developed in the early 1980s for *E. coli* (Tabor and Richardson 1985) and is based on the RNA polymerase of the bacteriophage T7 (T7 RNAP). This viral polymerase combines several features, such as high processivity and stringent selectivity towards its cognate promoter which makes it attractive for recombinant expression systems. Since then, it has been adopted to different Gram-negative and Gram-positive bacteria as well as to eukaryotic organisms (Brunschwig and Darzins 1992; Conrad *et al.* 1996; Elroy-Stein and Moss 1990).

3.1.1 Construction of a T7 RNA Polymerase Expression Vector

In this thesis a T7 RNA dependent expression system for *B. megaterium* was developed. For this purpose an expression plasmid allowing for *t7 rnap* expression in *B. megaterium* was constructed. It is based on plasmid pYZ5 and additionally carries the homologous xylose-inducible promoter and its regulatory elements. The novel expression plasmid, termed pMGBm19, was used for cloning of *t7 rnap*.

However, numerous attempts to clone *t7 rnap* under transcriptional control of the xylose-inducible promoter into plasmid pMGBm19 did not succeed. Since this promoter shows strong basal expression of the controlled genes in the cloning host *E. coli* (Jordan *et al.* 2007), production of T7 RNAP might be deleterious to *E. coli* in the early stage of plasmid establishment and cell recovery. Therefore, a new strategy was developed in order to clone an intact *t7 rnap* expression cassette. It takes advantage of the differing codon usage bias between the cloning host *E. coli* and the production host *B. megaterium*. While the base triplet GGA is the preferred codon for glycine in *B. megaterium* (Yang *et al.* 2007), it codes for merely 11 % of all glycine codons within all coding sequences of *E. coli*. Bearing in mind that a cluster of rare codons especially near the start site of a gene can strongly reduce the translational efficiency (Rosenberg

et al. 1993), the *t7 rnap* gene with four consecutive GGA codons at its 5'-end was cloned into the vector pMGBm19. Using this strategy, the construction of a functional *t7 rnap* expression cassette was successful. The resulting plasmid termed pT7-RNAP could further be used for efficient xylose-inducible expression of *t7 rnap* in *B. megaterium* (Figure 3.1).

3.1.2 Construction of a Vector for T7 RNA Polymerase Directed Expression of Target Genes

For T7 RNAP dependent recombinant gene expression, a plasmid comprising the T7 RNAP promoter ϕ10 and the Tϕ transcription terminator was constructed (Figure 3.1). The coding sequences for both elements were cloned into the plasmid pStop1622 – a derivative of pMM1522. Additionally, the resulting plasmid pP_{T7} has an optimised ribosomal binding site allowing for an efficient initiation of translation (Vellanoweth 1993). The multiple cloning site downstream of the ϕ10 promoter with 10 unique restriction enzyme cleavage sites allows for comfortable cloning of a gene of interest. The sequence of this multiple cloning site can also be found in all commercially available *B. megaterium* expression vectors (Mobitec GmbH, Göttingen, Germany) and therefore enables a simple and time-saving subcloning.

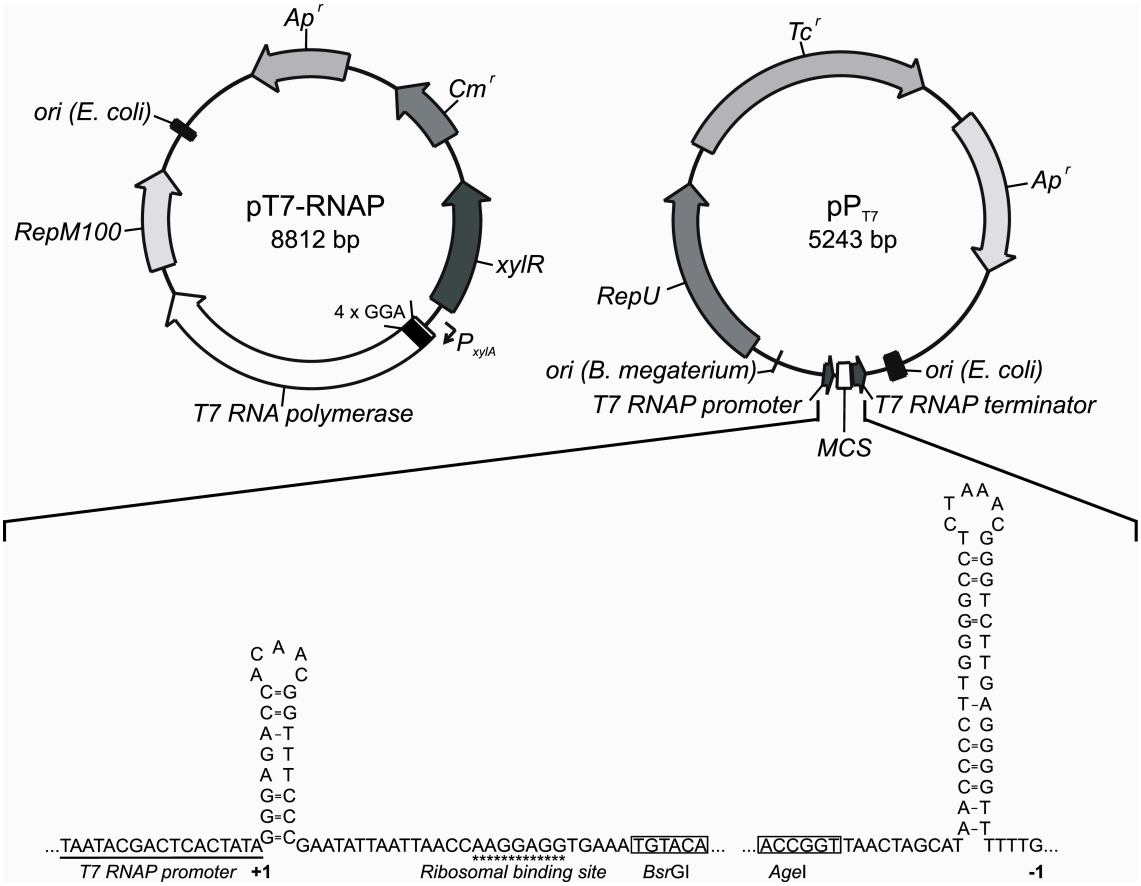

Figure 3.1: Structural elements of plasmids pT7-RNAP and pP$_{T7}$. Both plasmids are shuttle vectors which allow for positive selection and replication in *E. coli* and *B. megaterium*, respectively. The *Ap*^{r} gene confers resistance to ampicillin in *E. coli*. In *B. megaterium* chloramphenicol (*Cm*^{r}) can be used as selection marker in case of pT7-RNAP and tetracycline (*Tc*^{r}) in case of pP$_{T7}$. Due to the different nature of the replicons (*repU* and *repM100*) the plasmids can replicate in parallel in *B. megaterium*. Plasmid pT7-RNAP contains a 2,670 bp SpeI/XhoI fragment comprising *t7 rnap* and four consecutive GGA codons at the 5'-end under transcriptional control of P$_{xylA}$. Plasmid pP$_{T7}$ contains all structural elements necessary for a T7 RNAP dependent expression of target genes. The promoter sequence of T7 RNAP is underlined and the first nucleotide (+1) of the RNA transcript, as determined by Studier *et al.* (Studier *et al.* 1990), is indicated. The optimised ribosomal binding site (marked with stars) is followed by the multiple cloning site (*MCS*). The first and the last unique restriction enzyme sites are shown (marked with rectangles). The T7 RNAP transcription terminator represented by its potential stem loop structure is located downstream of the *Age*I site and the last nucleotide of the terminated RNA transcript (-1) is indicated.

3.1.3 T7 RNA Polymerase Dependent Production of Cytosolic Proteins

At first, the efficiency of the T7 RNAP dependent expression system for the production of cytosolic proteins in *B. megaterium* was evaluated. For this purpose GFP was used as model protein. The gene encoding GFP was cloned under transcriptional control of the T7 RNAP promoter into plasmid pP$_{T7}$. *B. megaterium* MS941 was cotransformed

with the resulting vector pP_{T7}-GFP and the plasmid pT7-RNAP. Expression analyses were performed in LB medium. To analyse the production of T7 RNAP, a western blot using a monoclonal T7 RNAP-specific antibody was performed. Before the addition of xylose no T7 RNAP was detected indicating the tight regulation of the homologous xylose-inducible promoter in *B. megaterium* (Biedendieck *et al.* 2007c). 1.5 h after induction of the *t7 rnap* expression, a strong band corresponding to the T7 RNAP appeared on the western blot. However, T7 RNAP levels did not stay constant over time. After reaching its maximum 3 h after induction of *t7 rnap* expression, the cellular amount of T7 RNAP declined steadily until the end of the cultivation (Figure 3.2 (A)).

The T7 RNAP dependent expression of *gfp* was monitored by analysing the soluble cytosolic proteins of equal amount of cells via SDS-PAGE. As expected, no GFP was detected before the addition of xylose. After the induction of *t7 rnap* expression, the appearance of a strong band with a relative molecular weight of ~27,000, corresponding to GFP, was detected on the SDS-PAGE gel. 1.5 h after induction of *t7 rnap* expression, GFP was already found to be the dominant cytosolic protein (Figure 3.2 (A)). However, similar to the decline in T7 RNAP production, the cellular amount of GFP started to decrease 3 h after induction of *t7 rnap* expression over time.

A different behaviour was observed when rifampicin was added into a culture of the plasmid strain *B. megaterium* MS941 carrying pT7-RNAP and pP_{T7}-GFP. Rifampicin is a selective inhibitor of bacterial DNA dependent RNA polymerases. Since the activity of bacteriophage originated T7 RNAP is not affected by this antibiotic, the addition of rifampicin directs the bacterial translational machinery exclusively towards the production of the recombinant protein whose gene is transcribed by T7 RNAP. The addition of rifampicin 1 h after induction of *t7 rnap* expression led to an abrupt entry into the stationary phase indicating a breakdown of the cellular metabolism (data not shown). In contrast to the reference culture without rifampicin treatment, analysis of T7 RNAP production via western blot showed a constant level of T7 RNAP over time. Similar to that, the cellular amount of recombinantly produced GFP stayed constant over time as estimated from the SDS-PAGE gel (Figure 3.2 (B)). Consequently, treatment with rifampicin seemed to block the synthesis of endogenous proteases and therefore elevated the stability of T7 RNAP and of the model protein GFP. However, these positive effects are contrasted by the observed growth defects induced by rifampicin.

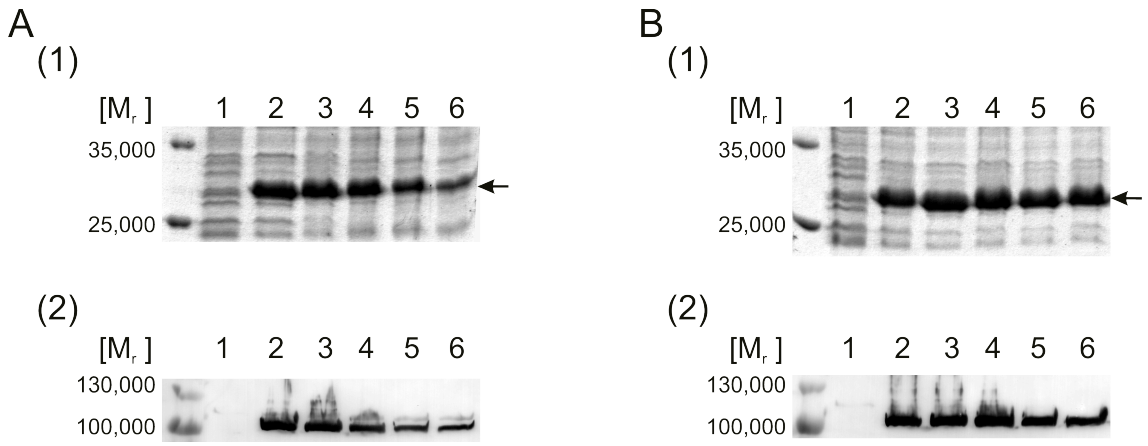

Figure 3.2: T7 RNA polymerase dependent cytosolic production of GFP. *B. megaterium* MS941 cotransformed with the plasmids pT7-RNAP and pP_{T7}-GFP was cultivated in LB medium at 37°C. Expression of *t7 rnap* was induced at an OD_{578} of 0.4 by the addition of 0.5 % (w/v) xylose. Cultivations were performed without (**A**) and with (**B**) the addition of rifampicin (200 µg ml^{-1}) which was added to the culture 1 h after induction of recombinant gene expression. Samples were taken before (lane 1), 1.5 h (lane 2), 3 h (lane 3), 4.5 h (lane 4), 6 h (lane 5) and 7.5 h (lane 6) after addition of xylose. (**1**) Soluble proteins of 5 x 10^8 cells were separated via 12 % SDS-PAGE and visualised by Coomassie Brilliant Blue. Arrows indicate protein bands representing GFP. (**2**) Immunological detection of T7 RNAP within the soluble protein fractions of 5 x 10^8 cells was performed by western blotting. A T7 RNAP specific monoclonal antibody (Novagen, Madison, USA) was used as primary antibody.

3.1.4 Comparison of T7 RNA Polymerase Dependent- and Xylose-Inducible Promoter Systems for the Production of Cytosolic Proteins

So far, the best studied promoter in *B. megaterium* is the xylose-inducible one. All commercially available expression systems base on this tightly regulable promoter. It has been extensively used for the production of different recombinant proteins, like GFP (Biedendieck *et al.* 2007c), a mannitol dehydrogenase (Baumchen *et al.* 2007), a hydrolase (Yang *et al.* 2007) or a single chain antibody fragment in *B. megaterium* (Jordan *et al.* 2007).

To compare the productivity of the T7 RNAP dependent expression system with the xylose-inducible one, again GFP was taken as intracellular model protein. As reference plasmid strain, *B. megaterium* MS941 transformed with the plasmid pRBBm34 was used (Biedendieck *et al.* 2007b). This vector carries a copy of *gfp* under transcriptional control of the xylose-inducible promoter. Amounts of GFP produced in this plasmid strain were then compared with those in *B. megaterium* MS941 cotransformed with pT7-RNAP/pP_{T7}-GFP grown in the presence or absence of 200 µg ml^{-1} rifampicin. Using the xylose-inducible promoter for *gfp* expression, a maximum of 7.2 mg l^{-1} of

GFP were produced 4.5 h after the addition of xylose. By applying the T7 RNAP dependent gene expression system, 5.3 times more GFP (38.3 mg l^{-1}) was recombinantly produced if rifampicin was added to a growing culture and even up to 7 times more (49.7 mg l^{-1}) without rifampicin, respectively (Figure 3.3). In this study, the overall better GFP production per biomass observed for cultures with the addition of rifampicin (Figure 3.2) was lost to the low growth yields of the corresponding cultures. Furthermore, the productivity was enhanced more than 6 times (12.8 mg l^{-1} h^{-1} of GFP) in case of using the T7 RNAP dependent system compared to 2 mg l^{-1} h^{-1} of GFP if the xylose-inducible promoter was used for recombinant *gfp* expression.

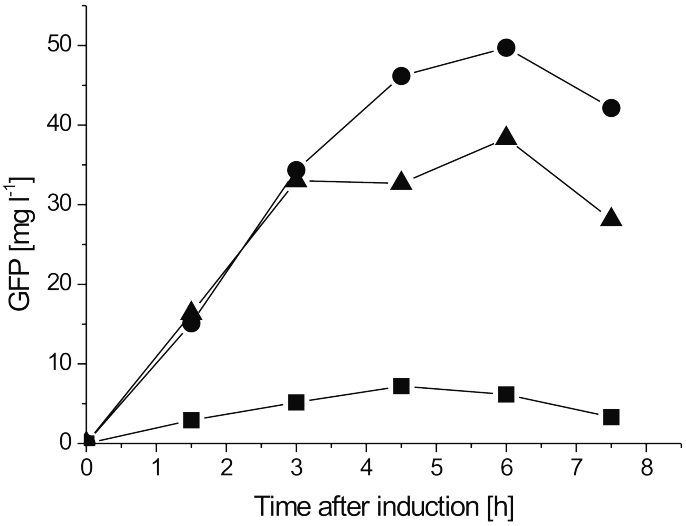

Figure 3.3: Amounts of heterologously produced GFP using different promoter systems. *B. megaterium* MS941 carrying the plasmid pRBBm34 (■) and *B. megaterium* MS941 cotransformed with the plasmids pT7-RNAP and pP_{T7}-GFP were cultivated in LB medium at 37°C. Recombinant gene expression was induced at an OD_{578} of 0.4 with 0.5 % (w/v) xylose. 1.5 h after xylose addition one culture of *B. megaterium* MS941 carrying the plasmids pT7-RNAP and pP_{T7}-GFP was supplemented with 200 µg ml^{-1} of rifampicin (▲) whereas the other was not (●). Samples were taken at indicated times and the intracellular amounts of GFP were measured spectrofluorometrically.

3.1.5 T7 RNA Polymerase Dependent Production of Secretory Proteins

The levansucrase LevΔ773 from *L. reuteri* 121 has already been extracellularly produced with *B. megaterium* by applying the xylose-inducible promoter (Biedendieck *et al.* 2007a). To test the transcriptional and translational efficiency of the T7 RNAP dependent expression system for the economic production of secretory proteins, heterologous LevΔ773 with a C-terminal His_6-tag fusion was used as model (here

referred to as LevΔ773). The plasmid strain *B. megaterium* MS941 carrying pRBBm15 was used as reference (Biedendieck *et al.* 2007a). It carries a vector-encoded copy of *levΔ773* fused with the coding sequence for a C-terminal His_6-tag transcribed under control of the xylose-inducible promoter. For translocation of the protein via the Sec-pathway, the gene was fused to the coding sequence for the N-terminal signal peptide of the homologous esterase LipA. The identical gene fusion was cloned into the plasmid pP_{T7} under transcriptional control of the ϕ10 promoter. The plasmid strain *B. megaterium* MS941 carrying pT7-RNAP was transformed with the resulting plasmid pP_{T7}-Lev.

Expression analyses were performed in LB medium. Comparison of extracellular protein patterns of both strains showed less LevΔ773 within the culture supernatant of *B. megaterium* MS941 carrying pT7-RNAP/pP_{T7}-Lev compared to *B. megaterium* MS941 transformed with pRBBm15 as determined via SDS-PAGE (Figure 3.4 (B)). Measurement of the volumetric levansucrase activity of the cell-free culture supernatants confirmed these results: Eight hours after induction of recombinant gene expression 48 U l^{-1} levansucrase were detected in the extracellular protein fraction of *B. megaterium* MS941 carrying pRBBm15 whereas by using the T7 RNAP dependent expression system for the secretory production of LevΔ773, a 3.2-fold decrease in volumetric levansucrase activity (15 U l^{-1}) was observed (Figure 3.4 (A)). Obviously, limitations affiliated with the translocation process seem to be responsible for this diminished T7 RNAP dependent production of LevΔ773.

The high level transcription and -formation of LevΔ773 might cause severe secretion stress in *B. megaterium* and thereby lead to the induction of stress responses. Responses to secretion stress have been extensively studied in *B. subtilis*. It could be shown that protein-misfolding at the membrane-cell wall interface in this organism results in the activation of the two component regulatory system CssR-CssS (Hyyrylainen *et al.* 2001) which in turn induces the formation of the membrane bound serine proteases HtrA and HtrB (Lulko *et al.* 2007). The presence of homologous genes coding for CssR-CssS and the membrane bound protease HtrA could be computationally predicted in *B. megaterium* DSM319. Immunological detection of LevΔ773 via its His_6-tag revealed that unprocessed LevΔ773 accumulated to significant higher amounts in the cell-associated protein fraction of *B. megaterium* MS941 cotransformed with pT7-RNAP/pP_{T7}-Lev compared to *B. megaterium* MS941 carrying pRBBm15 (Figure 3.4 (C)). Thus, LevΔ773 is inefficiently released from the translocation pore and might form improperly folded intermediates at the membrane-cell wall interface. By using rifampicin as an inhibitor of the bacterial protein

synthesising machinery, higher amounts of LevΔ773 could be secreted into the culture supernatant indicating a possible block of the stress response and further of the synthesis of proteases involved in the quality control of extracellular proteins.

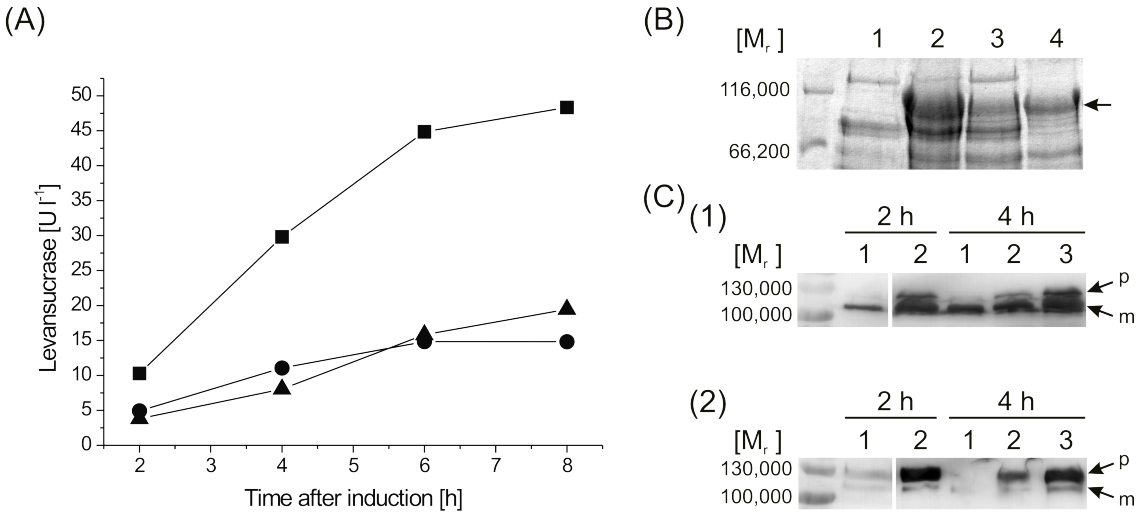

Figure 3.4: Secretory production of recombinant levansucrase LevΔ773. The *B. megaterium* MS941 plasmid strain pRBBm15 (■) and *B. megaterium* MS941 cotransformed with the plasmids pT7-RNAP and pP_{T7}-Lev were cultivated in LB medium at 37°C. The recombinant gene expression was induced at an OD_{578} of 0.4 with 0.5 % (w/v) xylose. 1.5 h after xylose addition, one culture of *B. megaterium* MS941 bearing the plasmids pT7-RNAP and pP_{T7}-Lev was treated with 200 µg ml^{-1} of rifampicin (▲) while the second culture was not (●). (**A**) The levansucrase activity within the cell-free growth medium was determined using the D.N.S. method. One unit was defined as the amount of enzyme releasing 1 µmol glucose per minute, describing the transferase and hydrolase activity. (**B**) 6 h after induction of recombinant gene expression proteins of 1.5 ml cell-free growth medium were precipitated with 44 % (w/v) ammonium sulphate. Proteins were separated via 12 % SDS-PAGE gels and visualised with Coomassie Brilliant Blue. Lane 1: *B. megaterium* MS941 (negative control), lane 2: *B. megaterium* MS941 carrying pRBBm15, lane 3: *B. megaterium* MS941 carrying pT7-RNAP/pP_{T7}-Lev, lane 4: *B. megaterium* MS941 carrying pT7-RNAP/pP_{T7}-Lev treated with rifampicin. The arrow indicates protein bands corresponding to heterologous LevΔ773. (**C**) Soluble (**1**) and insoluble (**2**) protein fractions of *B. megaterium* MS941 carrying the plasmid pRBBm15 and *B. megaterium* MS941 cotransformed with the plasmids pT7-RNAP and pP_{T7}-Lev were analysed via western blotting. Fractioned proteins of 5 x 10^8 cells were loaded onto each lane. His_6-tagged LevΔ773 was immunologically detected using a monoclonal His_6-specific primary antibody (Amersham). Lane 1: *B. megaterium* MS941 carrying pRBBm15, lane 2: *B. megaterium* MS941 carrying pT7-RNAP/pP_{T7}-Lev, lane 3: *B. megaterium* MS941 carrying pT7-RNAP/pP_{T7}-Lev treated with 200 µg ml^{-1} rifampicin 1.5 h after induction of *t7-rnap* expression. The indicated times represent the times after induction of recombinant gene expression. The positions of the premature (**p**) and mature (**m**) levansucrase are indicated.

The results indicate that an elevated expression of target genes, whose gene products are targeted to secretion, does not necessarily result in higher yields of extracellular proteins. Instead, fine-tuning in gene expression is necessary to prevent an overload of the secretion machinery and thereby the activation of the secretion stress response.

3.2 Codon Usage and Recombinant Protein Production in *Bacillus megaterium*

The choice of a suitable promoter is essential for the high-level transcrition of a gene of interest. But besides this transcriptional activity, the codon composition of a gene, aimed for heterologous expression strongly influences the translational efficiency and thus the overall protein yield. This phenomenon is caused by the degenerate nature of the genetic code which means that the majority of amino acids are encoded by more than one codon. In most cases such so-called synonymous codons just differ by one nucleotide in the third codon position. Synonymous codons are not used with equal frequencies and their usage varies between organisms and even between genes within the same genome.

3.2.1 Influence of Codon Clusters on Recombinant Gene Expression

The preference for distinct codons within a genome can be correlated with the abundance of tRNA species (Ikemura 1981). Experimental studies in *E. coli* revealed that mRNAs containing high portions of rare codons are less efficiently translated than mRNAs composed of preferred codons (Sorensen *et al.* 1989). In 1993, Rosenberg and colleagues demonstrated that consecutive rare codons near the 5'-end of a gene can substantially reduce the rate of protein synthesis (Rosenberg *et al.* 1993). Additionally, it was shown that clusters of rare AGG codons, encoding the amino acid arginine, lead to frameshift errors and premature translational termination in *E. coli* (Rosenberg *et al.* 1993).

Today, the increasing availability of whole genome sequence data enables the systematic analysis of codon usage bias in different organisms. Based on the genome sequence of *B. megaterium* the relative abundance of codons encoding a particular amino acid within all predicted open reading frames was calculated (Grote, 2008, unpublished results). Especially, codons for the amino acids alanine (GCC), arginine (CGG, AGG), glycine (GGG), leucine (CUC), serine (UCC) and threonine (ACC) turned out to be of rare use (Figure 3.6 (A)). In order to evaluate the effect of these rare codons on the synthesis of recombinant proteins in *B. megaterium*, a codon test system was established. It makes use of the green fluorescent protein (GFP). This marker protein can be easily quantified by fluorescence measurements even within cells. Further, its gene has a codon composition which is well suited for high-level expression in *B. megaterium* (Biedendieck *et al.* 2007c).

In a first step, the codon test system was generated. For this purpose the gene encoding GFP was amplified by PCR. The oligonucleotide primers were designed in such a way that the 5'-end of *gfp* was fused to four identical consecutive codons of interest. The PCR products were introduced individually into the *B. megaterium* expression vector pMM1522 (Biedendieck 2007) under transcriptional control of the xylose-inducible promoter (Figure 3.5).

Figure 3.5: Genetic elements of the versatile codon test plasmid. The -35 and -10 regions of the xylose-inducible promoter are underlined. Additionally, the transcription start site as determined by Rygus *et al.* is marked by "+1" (Rygus *et al.* 1991). The ribosomal binding site as well as the start site of the *gfp* open reading frame is indicated. Clusters of four identical consecutive codons were fused in frame to the coding region of GFP.

Expression analyses were performed in LB medium using the *B. megaterium* strain MS941 individually transformed with the different test plasmids. The amount of GFP was measured spectrofluorometrically 6 h after induction of *gfp* expression. For each amino acid mentioned above the impact of the predicted rare codons on the translation of *gfp* was determined by calculating relative GFP production yields. Relative expression levels for predicted rare codons were calculated by comparing the expression of *gfp* with a cluster of the most frequently used codon to the one of predicted rare codons.

The results of the expression analyses are presented in Figure 3.6. The cluster of four consecutive CUC codons, coding for the amino acid leucine, had the strongest influence on GFP production. Compared to the reference with a cluster of four UUA codons an 84 % reduction in GFP production was observed. Two codons were predicted to be rare in case of the amino acid arginine. The codons CGG and AGG turned out to limit the translational efficiency 58 and 76 %, respectively. In addition to fluorometric analysis, the inhibitory effect of corresponding codon clusters on GFP formation was exemplarily demonstrated by SDS-PAGE for codons coding for arginine (Figure 3.6 (B)). Overall, the tested rare codon clusters had a significant impact on GFP formation. The reduction in production yields ranged from 40 % up to 84 %.

(A)

Amino Acid	Codon	Codon Frequency	ΔG [kcal/mol]	Relative GFP Production
Alanine	GCU	36.5%	-5.4	1.00
	GCC	11.8%	-2.3	0.59 ± 0.10
Arginine	CGU	27.7%	-3.8	1.00
	CGG	5.8%	-7.2	0.24 ± 0.01
	AGG	6.5%	-2.9	0.42*
Glycine	GGA	41.5%	-6.5	1.00
	GGG	13.1%	-6.4	0.39 ± 0.02
Leucine	UUA	37.3%	-1.8	1.00
	CUC	5.1%	-0.5	0.16 ± 0.04
Serine	UCA	23.7%	-1.3	1.00
	UCC	7.9%	-0.5	0.18 ± 0.02
Threonine	ACA	40.6%	-0.5	1.00
	ACC	11.5%	-1.4	0.51 ± 0.06

(B)

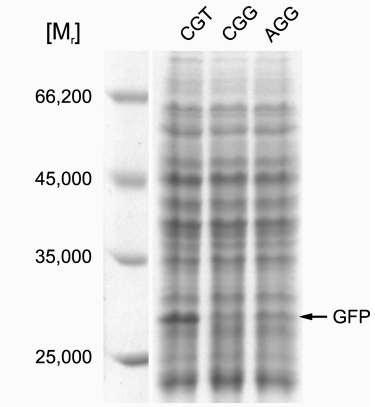

Figure 3.6: Effects of individual codon clusters on recombinant *gfp* expression. (**A**) The frequency of a codon within the *B. megaterium* genome was determined by analysing all predicted open reading frames. A cluster of four identical consecutive codons encoding for the indicated amino acids was fused to the 5'-end of *gfp* via PCR. The constructs were cloned under transcriptional control of the xylose-inducible promoter into the shuttle vector pMM1522. The *B. megaterium* strain MS941 was transformed with the resulting plasmids. Expression analyses were performed in LB medium at 37°C. Expression of *gfp* was induced at an OD_{578} of 0.4 by the addition of 0.5 % (w/v) xylose. The amount of GFP was measured spectrofluorometrically 6 h after the addition of xylose. Relative production yields were calculated for each amino acid by comparing the GFP formation of *B. megaterium* employing the codon test plasmid with either a cluster of the most frequently used codon or a cluster of predicted rare codons fused to the 5'-end of *gfp*. With exception for a cluster of four AGG codons (marked with "*"), data of two independent cultivations were used for the determination of relative production yields. The thermodynamic stabilities of potential secondary structures of the individual transcripts at 37°C were calculated from nucleotide -4 to +37 relative to the translation initiation site using the program "mfold" (Zuker 2003). (**B**) Production of GFP fused to a N-terminal cluster of four arginines was analysed via SDS-PAGE. The recombinant *B. megaterium* plasmid carrying strains were cultivated as indicated above. 6 h after induction of recombinant gene expression the soluble intracellular protein fractions were prepared and subsequently separated via 12 % SDS-PAGE. Proteins equivalent to approximately 5 x 10^8 cells were loaded per lane and stained with Coomassie Brilliant Blue after electrophoresis. The respective codons encoding for the cluster of consecutive arginines are indicated. Protein bands corresponding to the GFP fusion protein are marked by an arrow.

The altered production yields resulting from the fusion of codon clusters to the 5'-end of *gfp* could be due to several possible mechanisms. The design of the experiment eliminated posttranslational factors such as instabilities of the fusion proteins as explanation for the observed differences in GFP production since exclusively the quantities of identical fusion proteins were compared with each other. On the other hand, changes in the secondary and tertiary structure of the modified nucleotide sequence near the 5'-end of *gfp* might have an influence on the initiation of translation. Multiple studies showed that stable secondary structures near the 5'-end of a transcript can severely interfere with protein translation initiation (De Smits and van Duin 1990;

Griswold *et al.* 2003; Kudla *et al.* 2009). To elucidate the inhibitory influence of potentially stable mRNA secondary structures on the rate of translation, analysis on the thermodynamic stabilities of all transcripts was performed using the program “mfold” (Zuker 2003). The stability of the mRNA from nucleotide -4 to +37 (Kudla *et al.* 2009) relative to the translation initiation site was analysed (Figure 3.6 (A)). The data indicate that there is no strong correlation between the predicted thermodynamic stabilities and the observed differences in *gfp* expression levels. However, the frequency of a codon for a given amino acid within all open reading frames of the *B. megaterium* genome agrees well with relative GFP production levels. Thus, the pool of adequate tRNAs within the cell seems to determine the rate of GFP formation. If the number of test mRNAs being translated became larger than the number of individual tRNA molecules the ribosomal complex might stall at the site of rare codon clusters and thereby limit the formation of GFP. This observation therefore contradicts recent findings by Kudla *et al.* who argued that not codon bias but mRNA folding near the ribosomal binding site *i.e.* the rates of translation initiation play a predominant role in shaping expression levels (Kudla *et al.* 2009).

3.2.2 Computational Prediction of tRNA Genes

The increase of whole genome sequence information, demands bioinformatical tools for their functional annotation. Different algorithms have been developed for the prediction of tRNA genes (Fichant and Burks 1991; Lowe and Eddy 1997; Marvel 1986). The most prominent one is “tRNAscan-SE”. This tool is described as being able to identify 99-100 % of tRNA genes in DNA sequences while giving less than one false positive per 15 gigabases (Lowe and Eddy 1997). Due to this accuracy, it has been used for the prediction of tRNA genes in the *B. megaterium* strain DSM319.

Table 3.1 gives an overview of the computationally predicted tRNA species and their corresponding gene copy numbers. In total, 125 tRNA genes were identified belonging to 29 anticodon species. In contrast to *E. coli* which encodes 41 different anticodon species, most codons in *B. megaterium* are translated by a single anticodon. Isoacceptors just occur for the amino acids alanine (2), arginine (2), glycine (2), leucine (4), serine (3), threonine (2) and valine (2). Like in *E. coli* and *B. subtilis*, the majority of *B. megaterium* tRNA genes are highly clustered. Among all predicted genes only 8 exist as a single gene. The remaining 117 are organised in polycistronic operons.

Table 3.1: tRNA genes of *Bacillus megaterium* DSM319. The tRNA genes were predicted using "tRNAscan-SE" (Lowe and Eddy 1997).

Amino Acid Acceptor Group	Unmodified Anticodon	Number of tRNA Genes
Alanine	GGC	1
	UGC	5
Arginine	ACG	5
	GCG	1
Asparagine	GUU	8
Aspartic Acid	GUC	6
Cysteine	GCA	1
Glutamine	UUG	4
Glutamic Acid	UUC	8
Glycine	GCC	6
	UCC	4
Histidine	GUG	4
Isoleucine	GAU	3
Leucine	GAG	1
	UAG	5
	CAA	1
	UAA	3
Methionine	CAU	13
Phenylalanine	GAA	4
Proline	UGG	3
Serine	GGA	1
	UGA	5
	GCU	3
Threonine	GGU	1
	UGU	7
Tryptophan	CCA	3
Tyrosine	GUA	4
Valine	GAC	1
	UAC	7

In total, *E. coli* has 79 tRNA genes which all encode a conserved CCA-sequence at the 3'-end of the tRNA acceptor stem. This motive is of general importance for the recognition of tRNAs by their aminoacyl-tRNA-synthetase counterparts and for tRNA movement in the ribosome during translation. By contrast, 18 % of all predicted *B. megaterium* tRNA genes lack this motive. This characteristic has also been observed in *B. subtilis* (Wen *et al.* 2005). Lack of a CCA-motive in tRNA genes therefore points to the presence of a CCA-adding enzyme in *B. megaterium*. Such an enzyme, termed *papS*, was already identified and functionally characterised in *B. subtilis* (Raynal *et al.* 1998). Indeed, computational analysis showed that the *B. megaterium* strain DSM319 encodes a homologue to PapS of *B. subtilis*.

Interestingly, the experimental identification of rare codons in *B. megaterium* (chapter 3.2.1) is in good agreement with the gene copy number of corresponding tRNA genes. Most tRNAs decoding rare codons are represented by just a single chromosomal gene copy (Table 3.2). Analysis of intracellular tRNA levels in *E. coli* showed that besides

promoter efficiencies, processing of tRNA precursors and tRNA stabilities, the gene copy number of tRNA species mainly influences the abundance of corresponding tRNAs within the cell (Inokuchi and Yamao 1995). The presented results indicate that this also seems to apply for *B. megaterium*.

Table 3.2: Correlation between tRNA gene copy number and identity of rare codons in *Bacillus megaterium* DSM319. The glycine codon GGG is probably decoded by whobble basepairing since *B. megaterium* lacks a tRNA with the anticodon CCC (as predicted with "tRNAscan-SE") (Lowe and Eddy 1997).

Amino Acid	Rare Codon	Decoding tRNA Species	Gene Copy Number
Alanine	GCC	GGC	1
Glycine	GGG	UCC	4
Leucine	CUC	GAG	1
Serine	UCC	GGA	1
Threonine	ACC	GGU	1

3.2.3 Coexpression of a Rare tRNA Gene

Common methods to elevate the expression of a heterologous gene whose expression would be limited by the codon usage of the host cell are (I) the synthesis of a codon optimised gene or (II) the coexpression of tRNA genes decoding rare codons. The first strategy is a relatively novel invention and gained increasing importance since the market prices for gene synthesis dropped significantly over the last few years. On the other hand, coexpression of rare tRNA genes is long known to support the expression of recombinant genes which otherwise would be limited by the presence of many rare codons. In the year 2000, Stratagene (La Jolla, USA) filed a patent for enhanced expression of heterologous genes in *E. coli* by coexpressing all limiting tRNA genes. Today, different recombinant *E. coli* strains are marketed which carry additional plasmid-encoded rare tRNA genes under transcriptional control of their native promoter or a recombinant constitutive one.

In order to analyse the influence of tRNA coexpression on heterologous gene expression in *B. megaterium* the rare $tRNA^{Thr}_{GGU}$ was chosen as model. This tRNA is merely encoded by a single gene copy and does not lie within an operon of tRNA genes (chapter 3.2.2). Closer genetic studies indicated the presence of a putative transcription terminator downstream of its coding region (Figure 3.7). Investigations on the influence of coexpressed $tRNA^{Thr}_{GGU}$ on the expression of *gfp* with a cluster of corresponding rare ACC codons demanded a plasmid which can stably coexist in *B. megaterium* in the presence of pMM1522 derivatives. Plasmid pYZ5, a derivative of pYZ11 (Kunnimalaiyaan *et al.* 2001)), exhibits such characteristic. It is a shuttle vector

carrying genetic elements for replication and selection in *E. coli* and *B. megaterium*. Replication in *B. megaterium* is dependent on the rolling circle replicon derived from plasmid pBM100 of *B. megaterium* QM B1551 (Kunnimalaiyaan *et al.* 2001).

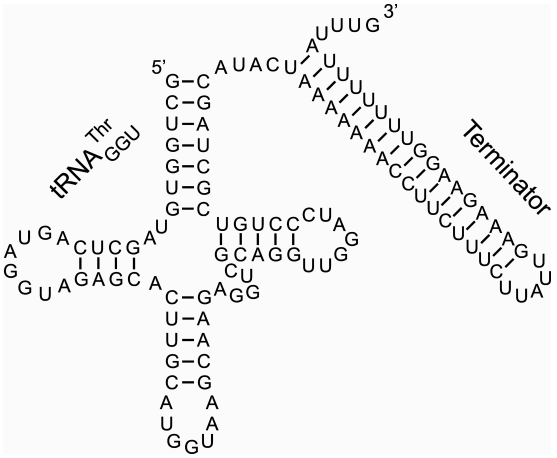

Figure 3.7: Secondary structure of computationally predicted tRNA $^{Thr}_{GGU}$ and its putative terminator. "tRNAscan-SE" was used for the identification of tRNA $^{Thr}_{GGU}$ (Lowe and Eddy 1997). Its potential secondary structure as well as the one of its putative transcriptional terminator at 37°C was predicted using "mfold" (Zuker 2003).

The computationally predicted tRNA $^{Thr}_{GGU}$ including its putative promoter region and the transcriptional terminator were amplified by PCR and cloned into the plasmid pYZ5. *B. megaterium* MS941 was cotransformed with the resulting vector ptRNA $^{Thr}_{GGU}$ and the expression vector pMGBm96, carrying *gfp* fused to a cluster of the four corresponding rare ACC codons. *B. megaterium* cotransformed with this *gfp*-carrying vector and pYZ5-P_{spac} (derivative of pYZ5) was used as reference. Expression analyses were performed in LB medium and the amount of GFP was measured fluorometrically.

Compared to the reference without additional tRNA $^{Thr}_{GGU}$ expression, plasmid-encoded supplementation of tRNA $^{Thr}_{GGU}$ led to a 40 % increase in GFP production 3 h after induction of recombinant *gfp* expression (Figure 3.8). Thus, computationally predicted tRNA $^{Thr}_{GGU}$ encodes a functional tRNA which can decode the rare ACC codon. Thereby, it can elevate translation of genes which is otherwise limited by the presence these codons. This observation emphasises the general feasibility of tRNA coexpression on elevating recombinant protein production in *B. megaterium*.

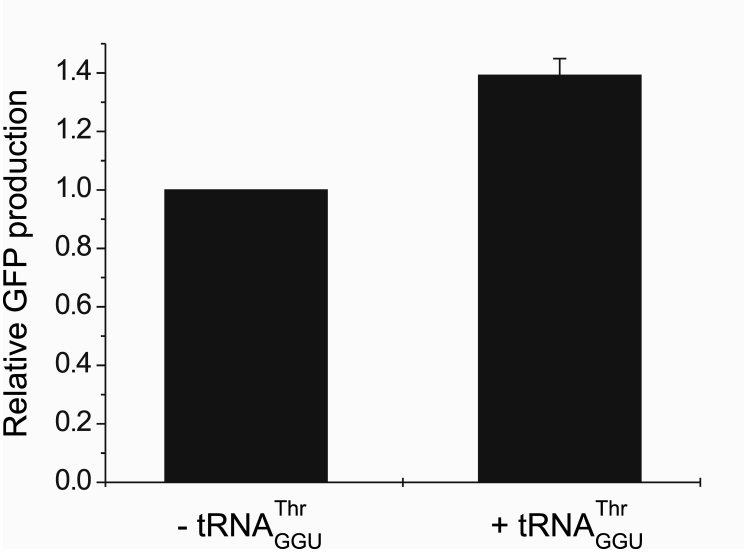

Figure 3.8: Influence of tRNA$^{Thr}_{GGU}$ coexpression on recombinant *gfp* expression. The functionality of computationally predicted tRNA$^{Thr}_{GGU}$ was tested by expressing *gfp* with a cluster of four consecutive ACC codons with or without plasmid-encoded supplementation of tRNA$^{Thr}_{GGU}$. For this purpose, the corresponding *B. megaterium* MS941 plasmid strains were cultivated in LB medium at 37°C. Recombinant *gfp* expression was induced at an OD_{578} of 0.4 by the addition of 0.5 % (w/v) xylose. The amount of GFP was measured spectrofluorometrically 3 h after the addition of xylose. GFP production levels with the supplementation of tRNA$^{Thr}_{GGU}$ (+tRNA$^{Thr}_{GGU}$) were set in relation to those without tRNA$^{Thr}_{GGU}$ coexpression (-tRNA$^{Thr}_{GGU}$).

3.3 Development of Natural Competence in *Bacillus megaterium*

Up to now *B. megaterium* has to be laboriously genetically manipulated by conventional Campbell-type recombination. Targeted gene-knockouts generated by single crossover events can be obtained with the help of plasmids with temperature sensitive origins of replication (Barg 2003). However, these mutants are unstable since the mutated allele can reconvert into the wild-type by a random second crossover event. Counterselectable markers which allow for the positive selection of mutants which have undergone the second crossover event greatly enhance the speed for the isolation of defined mutants (Reyrat *et al.* 1998). Unfortunately, such markers have not been proven for *B. megaterium*, so far. Consequently, the inactivation of a single chromosomal gene involves screening of hundreds of clones and can last several months.

The active acquisition of exogenous DNA and its site-specific integration into the chromosome by means of natural competence on the other hand represents a valuable

tool for genetic manipulation in *B. subtilis*. Similarly, introduction of transforming DNA into natural competent *B. megaterium* would greatly simplify the rational design of improved strains for recombinant protein production. However, the development of natural competence in *B. megaterium* has not been described so far.

All following computational analyses and experiments were performed with the *B. megaterium* type strain DSM319. To simplify matters, this strain is refered to as *B. megaterium* in the following chapter.

3.3.1 Genetic Prerequisites for the Development of Natural Competence

Due to the recently available genome data of *B. megaterium*, detailed *in-silico* analyses could be performed elucidating its physiological capabilities. BLAST analyses for the presence of essential genes involved in the development of natural competence led to the identification of a homologue to the central transcription factor ComK (here refered to $ComK_{Bmeg}$). Moreover, homologues to almost all genes known to be involved in DNA-uptake and recombination during natural competence could be annotated in the *B. megaterium* genome (Table 3.3). *B. megaterium* only lacks the gene coding for a homologue to ComFB. ComFB has no similarity to other proteins and until today its precise function remains unclear. Studies in which *comFB* was inactivated by targeted gene-knockout indicated that the corresponding protein is not essential for the development of natural competence in *B. subtilis* 168 (Londono-Vallejo and Dubnau 1993). This observation is supported by recent results with *Bacillus cereus* ATCC14579. Although this strain also lacks *comFB* it develops a functional machinery for DNA-binding, -uptake and -recombination (Mironczuk *et al.* 2008). Thus, *B. megaterium* may exhibit all genetic prerequisites which are essential for the formation of a functional DNA transformation machinery.

Table 3.3: Computational prediction of proteins in *Bacillus megaterium* homologous to proteins of *Bacillus subtilis* 168 involved in DNA uptake and recombination during natural competence. BLAST analyses were performed with *B. subtilis* 168 protein sequences against translated putative proteins of *B. megaterium* DSM319 stored in the database MEGABAC.

Protein	Function	ORF ID in MEGABAC	E-Value
ComK	Competence transcription factor	4700	$4xe^{-08}$
ComC	Involved in DNA-binding	3463	$7xe^{-43}$
ComEA	Involved in DNA binding and translocation	3406	$1xe^{-38}$
ComEB	Similar to dCMP deaminase	3418	$6xe^{-97}$
ComEC	Involved in DNA-binding and translocation	3432	$1xe^{-34}$
ComFA	Involved in DNA-translocation	4546	$1xe^{-32}$
ComFB	Involved in DNA-translocation	-	-
ComFC	Involved in DNA-translocation	4544	$3xe^{-40}$
ComGA	Involved in DNA-binding	3314	$1xe^{-126}$
ComGB	Involved in DNA-binding	3312	$1xe^{-37}$
ComGC	Involved in DNA-binding	3313	$3xe^{-16}$
ComGD	Involved in DNA-binding	4589	0.53
ComGE	Involved in DNA-binding	3321	$4xe^{-4}$
ComGF	Involved in DNA-binding	3315	$7xe^{-7}$
ComGG	Involved in DNA-binding	3317	0.037
RecA1	Multifunctional SOS repair regulator	231	$1xe^{-156}$
RecA2	Multifunctional SOS repair regulator	469	$1xe^{-139}$
NucA	Nuclease	2042	$1xe^{-43}$
Nin	Inhibitor of NucA	100	$3xe^{-30}$
YwpH	Similar to single-strand DNA-binding protein	5210	$3xe^{-38}$
RadC	Similar to DNA-repair protein	3466	$3xe^{-90}$
AddA	ATP-dependent deoxyribonuclease	4012	0.0
AddB	ATP-dependent deoxyribonuclease	4013	0.0
Smf	DNA processing	787	$6xe^{-64}$
YjbF	No similarity to other proteins	3998	$6xe^{-42}$
BdBC	Thiol-disulfide oxidoreductase	1310	$1xe^{-35}$
BdbD	Thiol-disulfide oxidoreductase	1313	$6xe^{-28}$

3.3.2 Computational Prediction of ComK-Binding Sites

The development of natural competence is a complex process which is tightly regulated and involves the activation of more than 100 genes. ComK represents the central transcription factor for the development of natural competence in *B. subtilis* 168 (van Sinderen *et al.* 1995). Formation of ComK above a certain threshold leads to the transcriptional activation of a complex set of late-competence genes whose gene products mediate binding, processing and internalization of transforming DNA.

In *B. subtilis* 168 ComK funtions as a tetramer composed of two dimers. Each dimer recognises the consensus sequence $A_4N_5T_4$ also called AT-box (Hamoen *et al.* 1998). The distance between two AT-boxes can vary between one, two or three helical turns.

None of these called K-boxes lying upstream of the late competence genes match the consensus sequence perfectly and K-boxes with deviations of up to 3 bp are still recognised by ComK (Hamoen *et al.* 2002).

To prove if K-boxes are present in the upstream regions of putative late competence genes in *B. megaterium* its genome sequence was analysed computationally. For this purpose the software Virtual Footprint (Münch *et al.* 2005) was applied. This tool is able to detect binding sites of transcription factors by searching for matches between the calculated position weight matrix of the transcription factor motif and a given nucleotide sequence.

Table 3.4 gives an overview about the results. The finding that only a fraction of homologues to late competence genes in *B. megaterium* has K-boxes within their putative promoter regions is contrary to the situation in *B. subtilis* 168 where each of the respective promoter regions possesses a K-box (Hamoen *et al.* 2002). This observation is puzzling since the presence of a K-box within the promoter region is essential for the recognition and activation by ComK.

Table 3.4: Prediction of ComK binding sites in *Bacillus megaterium*. K-boxes within putative promoter regions of genes known to be activated by ComK in *B. subtilis* 168 were predicted with Virtual Footprint (Münch *et al.* 2005).

Gene	Operon	Function	K-Box Present
comK	-	Competence transcription factor	no
comC	-	Involved in DNA-binding	yes
comEA	*comE*	Involved in DNA binding and translocation	yes
comEB	*comE*	Similar to dCMP deaminase	yes
comEC	*comE*	Involved in DNA-binding and translocation	yes
comFA	*comF*	Involved in DNA-translocation	yes
comFC	*comF*	Involved in DNA-translocation	yes
comGA	*comG*	Involved in DNA-binding	no
comGB	*comG*	Involved in DNA-binding	no
comGC	*comG*	Involved in DNA-binding	no
comGD	*comG*	Involved in DNA-binding	no
comGE	*comG*	Involved in DNA-binding	no
comGF	*comG*	Involved in DNA-binding	no
med	-	Positive regulator of *comK*	no
comZ	-	Positive regulator of *comG*	yes
nucA	*nucA*	Nuclease	no
nin	*nucA*	Inhibitor of NucA	no
recA1	-	Multifunctional SOS repair regulator	yes
recA2	-	Multifunctional SOS repair regulator	yes
ywpH	-	Similar to single-strand DNA-binding protein	no

To test whether the putative promoter regions of homologues to late competence genes in *B. megaterium* show an altered consensus sequence for alternative

transcription factors an additional computational approach was done. For this purpose the bioinformatic tools BioProspector and MEME were applied (Liu *et al.* 2001; Bailey and Elkan 1994). Both programs allow for the detection of conserved motifs in upstream regions of coexpressed genes. They were applied indepently using the putative promoter regions of all genes listet in Table 3.4. However, the identification of an alternative transcription factor binding motif failed (data not shown) possibly due to the inadequate size of the data set.

3.3.3 Recombinant Overproduction of ComK

So far, no biological function based on experimental results has been assigned to ComK$_{Bmeg}$ in literature. The prediction of its cellular role solely relies on its homology to ComK from *B. subtilis* 168.

To further elucidate the function of ComK$_{Bmeg}$ *in vivo* the respective putative gene was cloned under transcriptional control of the xylose-inducible promoter into the plasmids pStop1622 and pN-His-TEV1622 (Biedendieck *et al.* 2007c). The resulting plasmids were termed pDFcomK$_{Bmeg}$ and pDFHis-comK$_{Bmeg}$, respectively (Figure 3.9). In case of pDFcomK$_{Bmeg}$ transcription from the xylose-inducible promoter resulted in the formation of ComK$_{Bmeg}$ with its native N-terminus. A translational fusion of ComK$_{Bmeg}$ with a N-terminal His$_6$-tag followed by a TEV-protease cleavage site yielded from xylose-inducible transcription from pDFHis-comK$_{Bmeg}$.

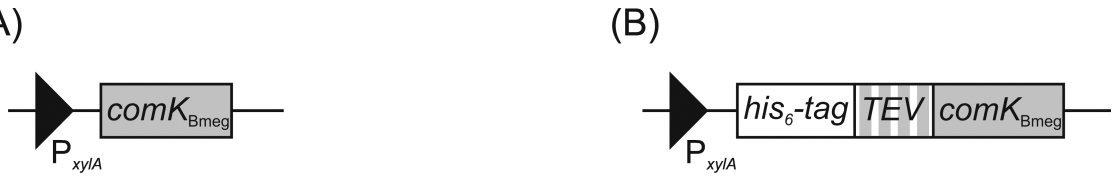

Figure 3.9: Genetic elements of plasmids (A) pDFcomK$_{Bmeg}$ and (B) pDFHis-comK$_{Bmeg}$. Putative *comK$_{Bmeg}$* from *B. megaterium* was cloned under transcriptional control of the xylose-inducible promoter P$_{xylA}$ into the plasmids pStop1622 and pN-His-TEV1622, respectively. Recombinant production resulted either in the formation of (A) ComK$_{Bmeg}$ or in the formation of (B) ComK$_{Bmeg}$ fused to a His$_6$-tag which can be cleaved off with the help of TEV-protease.

B. megaterium was transformed with each plasmid seperately and expression analyses were performed in minimal medium suited for the development of natural competence (Mironczuk *et al.* 2008). Fructose instead of glucose was used as carbon source in order to prevent a possible catabolite repression of the xylose-inducible promoter. Recombinant overexpression of *comK$_{Bmeg}$* was induced with varying concentrations of xylose and the formation of His$_6$-tagged ComK$_{Bmeg}$ was monitored via western blot using a monoclonal His$_6$-tag specific antibody.

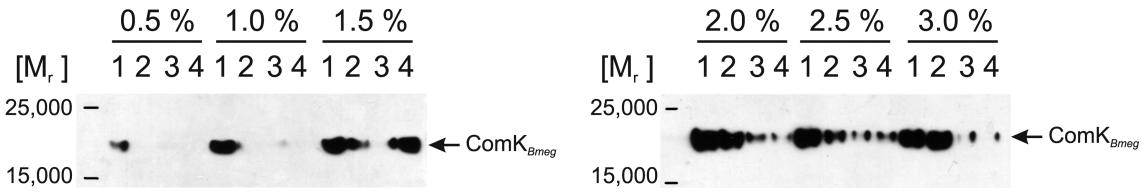

Figure 3.10: Recombinant overproduction of ComK$_{Bmeg}$. The *B. megaterium* plasmid strain pDFHis-comK$_{Bmeg}$ was cultivated at 37°C. Expression of *comK$_{Bmeg}$* was induced at an OD_{578} of 0.4 by the addition of 0.5 %, 1.0 %, 1.5 %, 2.0 %, 2.5 % and 3.0 % (w/v) xylose, respectively. Samples were taken 1.5 h (1), 3 h (2), 4.5 h (3) and 6 h (4) after induction of recombinant gene expression. Insoluble proteins of 5 x 10^8 cells were separated via 15 % SDS-PAGE and transferred onto a PVDF membrane. Recombinant ComK$_{Bmeg}$ fused to a His_6-tag was detected immunologically using a His_6-tag specific primary antibody (GE Healthcare, Piscataway, USA).

ComK$_{Bmeg}$ with a His_6-tag fusion could be sucessfully produced in *B. megaterium*. The level of recombinant overproduction could be fine-tuned by varying the concentration of the inducer xylose. Maximal induction of gene expression occurred using 2.0 % (w/v) xylose. Higher concentrations did not result in elevated amounts of ComK$_{Bmeg}$ (Figure 3.10).

ComK$_{Bmeg}$ was mainly detected within the insoluble intracellular protein fraction containing the cell debris and genomic DNA. This observation gives a first indication that computationally predicted ComK$_{Bmeg}$ might indeed represent a functional transcription factor which appears predominantly in association with its cognate recognition sequences *in vivo*.

3.3.4 Construction of GFP Reporter Gene Fusions

Transcriptional fusions of *gfp* with putative promoter regions of homologues to late competence genes in *B. megaterium* were constructed to prove their activation by ComK$_{Bmeg}$. For this purpose *gfp* was cloned into the shuttle vector pYZ5. The resulting vector pDFgfp was then used to construct promoter-*gfp* fusions. The upstream region of *recA1* as well as the one of the *comG*-operon from *B. megaterium* were chosen as representatives for ComK activated loci in *B. subtilis* 168. The selected upstream regions extended from the intergenic part into the upsteam neighbouring gene in order to be sure that they contain all regulatory elements which may be necessary for their activation. The corresponding plasmids were termed pDFrecA-gfp and pDFcomG-gfp, respectively. The plasmid strain *B. megaterium* pDF-comK$_{Bmeg}$ was transformed with each plasmid seperately.

3.3.5 Influence of ComK Overproduction on Cell-Morphology and Growth Behaviour

First expression analyses indicated that homologous overproduction of $ComK_{Bmeg}$ has significant influence on the growth behaviour of *B. megaterium*. Therefore, in a first step this remarkable feature was studied in more detail. For this purpose *B. megaterium* bearing the two plasmids pDFComK_{Bmeg} and pDFcomG-gfp was cultivated in shaking flasks using either minimal- or LB medium. Recombinant *comK*$_{Bmeg}$ expression was induced by the addition of xylose. The OD_{600} was measured over a period of approximately 7.5 h.

The growth characteristics differed significantly between cultures overproducing $ComK_{Bmeg}$ in comparison to reference cultures without the addition of the inducer xylose (Figure 3.11 (A)). In case of LB medium a massive breakdown in cell density occurred in a time-delayed manner after induction of *comK*$_{Bmeg}$ expression. The OD_{600} decreased over a period of approximately 3.5 h more than 2-fold from OD_{600} 2.52 to 1.13. This breakdown was not that prominent by using minimal- instead of LB medium. However, compared to the reference culture without recombinant overproduction of $ComK_{Bmeg}$ less than half of the final OD_{600} was reached.

Macroscopically, the overproduction of $ComK_{Bmeg}$ resulted in a clumpy appearance of the liquid culture (Figure 3.11 (B)). This phenomenon in conjunction with competence development has just recently been described for *Streptococcus pneumoniae*. Håvarstein and collegues demonstrated that it results from the presence of extracellular DNA liberated by lysed non-competent pneumococci within a mixed culture of competent and non-competent cells (Havarstein *et al.* 2006). The precise mechanism of this selective lysis of siblings, also called microbial fratricide, is not fully understood. What is known so far is that the major peptidoglycan hydrolases LytA and LytC as well as the late competence protein CbpD are involved in the lysis of non-competent *S. pneumonia* cells in liquid medium. It could be shown that LytA and LytC are provided by the non-competent target cells (Guiral *et al.* 2005). The putative amidase/peptidase CbpD on the other hand is exclusively produced by the competent attacker cells and is assumed to represent the trigger factor for the lytic action of LytA and LytC (Eldholm *et al.* 2009). In addition to CbpD, competent cells produce ComM which has been shown to protect competent *S. pneumoniae* cells against their own lysins (Havarstein *et al.* 2006).

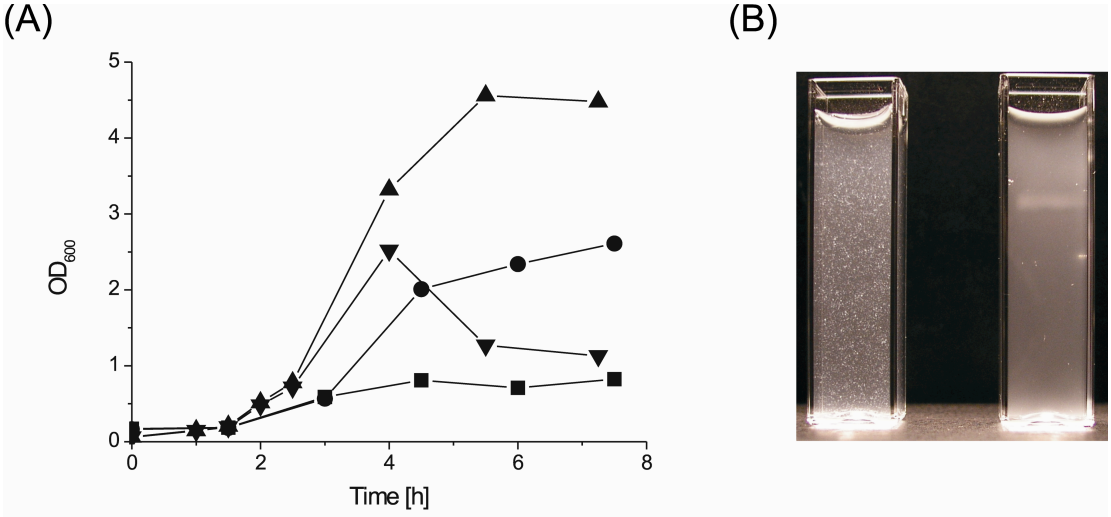

Figure 3.11: Influence of ComK$_{Bmeg}$ overproduction on cell growth. (A) The *B. megaterium* plasmid strain pDFcomK$_{Bmeg}$/pDFcomG-gfp was cultivated in shaking flasks at 37°C either in LB medium (▲ and ▼) or in minimal medium (● and ■). After reaching an OD_{600} of 0.6 – 0.8 one culture was supplemented with xylose to induce recombinant *comK*$_{Bmeg}$ expression. In case of LB medium xylose was added to a final concentration of 1 % (w/v) (▼) and in case of minimal medium to a final concentration of 2 % (w/v) (■). **(B)** The *B. megaterium* plasmid strain pDFcomK$_{Bmeg}$/pDFcomG-gfp was cultivated in shaking flasks in minimal medium at 37°C. After reaching an OD_{600} of 0.4 one culture was supplemented with 2 % (w/v) xylose whereas the second culture was left untreated. After a period of 5 h samples were taken, diluted in a ratio of 1:4 with minimal medium and applied to cuvettes. The left cuvette represents the culture overproducing ComK$_{Bmeg}$ and the right cuvette the culture without recombinant *comK*$_{Bmeg}$ expression.

Microscopic images of culture-samples emphasised the hypothesis that overproduction of ComK$_{Bmeg}$ in *B. megaterium* causes lysis of at least part of the population. A lot of cell debris was observed within the culture overproducing ComK$_{Bmeg}$ whereas this was not the case in the reference culture. Moreover, the cell morphology differed significantly. Overproduction of ComK$_{Bmeg}$ leads to cells being roughly half in size compared to cells from a culture without induction of recombinant *comK*$_{Bmeg}$ expression. They also exist no longer in chains but predominantly as single cells (Figure 3.12 (A)).

Since microscopic analyses include just a few cells of the bacterial population as a whole, additional flow cytometric studies were performed. This technique enables the characterisation of a very large number of cells with respect to their morphology. The relative size of a cell can be esimated by low angle light deflection which is proportional to its cross-sectional area. The relative narrow angle light scatter intensity is a measure for it (Rieseberg *et al.* 2001).

The narrow angle light scattering pattern differs significantly between the cultures with- and without ComK$_{Bmeg}$ overproduction (Figure 3.12 (B)). The largest fraction of cells

and cell debris within a population of *B. megaterium* pDFcomK$_{Bmeg}$ overproducing ComK$_{Bmeg}$ has a low relative narrow-angle light scatter. This characteristic contrasts the situation within the reference culture. Without ComK$_{Bmeg}$ overproduction the relative narrow-angle light scatter intensity of cells within the whole population is rather equally distributed. Thus, the results of the flow cytometric analyses quantitatively support the microscopic observations which indicated a strong influence of ComK$_{Bmeg}$ overproduction on size, morphology and integrity of individual *B. megaterium* cells within the whole population.

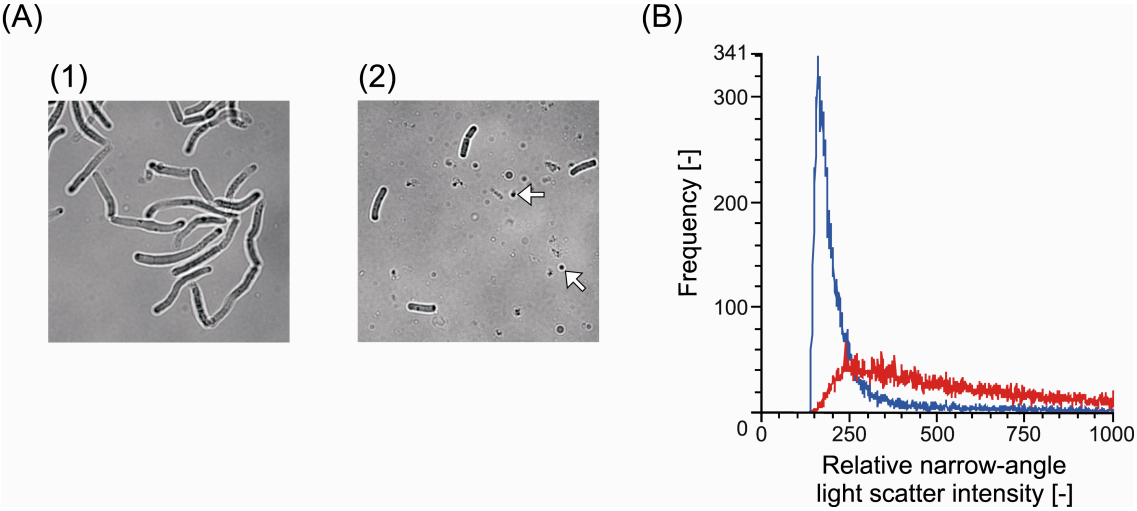

Figure 3.12: Morphological changes in cultures overproducing ComK$_{Bmeg}$. (A) Two cultures of the *B. megaterium* plasmid strain carrying pDFcomK$_{Bmeg}$/pDFcomG-GFP were cultivated in parallel in minimal medium at 37°C. One culture was supplemented with 2 % (w/v) xylose to induce recombinant *comK*$_{Bmeg}$ expression whereas the second culture was left untreated. After a cultivation period of 23 h microscopic images were taken of the **(1)** uninduced and the **(2)** induced culture. Both images were shot using a magnification of 1,000. Cell debris is exemplarily marked by arrows. **(B)** Instead of using 2 % (w/v) xylose to induce homologous *comK*$_{Bmeg}$ expression a concentration of 0.5 % (w/v) was applied. After a cultivation period of 4 h samples of the uninduced **(red line)** and the induced **(blue line)** culture were analysed via flow cytometry. In each case 25,000 particles (cells and cell debris) were measured with respect to their individual scattering of narrow-angle light.

Lysis of at least a fraction of cells within a culture overproducing ComK$_{Bmeg}$ could be due to several possible mechanisms. First, the situation in *B. megaterium* may be comparable to the one in *S. pneumoniae* where the competent cells produce lysins which cause the death of their non-competent counterparts leading to the liberation of transforming DNA. Second, overproduction of ComK$_{Bmeg}$ may cause autolysis of *B. megaterium*. This complete new mechanism has just recently been described for the oral pathogen *Streptococcus mutans* (Perry *et al.* 2009). Addition of high levels of the peptide pheromone competence-stimulating peptide (CSP) lead to the formation of natural competence in *S. mutans* but further also to autolysis of the competent cells.

Intracellular accumulation of the type II bacteriocin mutacin V (Csp) has been shown to be the major factor for this autolysis (Perry *et al.* 2009). It has been proposed that the biological function underlying this phenomenon is the dissemination of fitness-enhancing genes in oral biofilm under stress conditions.

To further analyse if overproduction of $ComK_{Bmeg}$ causes the formation or the liberation of some kind of extracellular lysin or of a factor which triggers lysis, the *B. megaterium* plasmid strain $pDFcomK_{Bmeg}$ and the reference strain *B. megaterium* pMM1520 were culivated in parallel in LB medium. 7 h after the addition of the inducer xylose the OD_{600} was determined to 4.28 for *B. megaterium* pMM1520 and to 1.33 for *B. megaterium* $pDFcomK_{Bmeg}$. Afterwards, the bacteria were pelleted by centrifugation. The culture supernatants were filtered through a sterile filter and added each to a final volume of 50 % (v/v) to an exponentially growing culture of *B. megaterium*. If overproduction of $ComK_{Bmeg}$ leads to the formation of extracellular lysins or of a trigger factor for lysis, supplementation of the cell-free culture supernatant of a $ComK_{Bmeg}$ overproducing culture should negatively influence the growth of a fresh culture of *B. megaterium*. However, this was not the case (Figure 3.13 (A)). Compared to the culture of *B. megaterium* which was supplemented with the cell-free culture supernatant of the reference strain, the addition of medium from the $ComK_{Bmeg}$ overproducing culture resulted in even higher cell densities.

On the other hand, microscopic analyses showed a clear influence of the origin of the supplemented culture supernatant on the cell morphology of the resulting population. *B. megaterium* cells which grew in a medium originated from a $ComK_{Bmeg}$ overproducing culture showed significantly smaller cells and less chain formation than bacteria within the reference culture (Figure 3.13 (B)). This observation is in analogy to the situation in a population of *B. megaterium* overproducing $ComK_{Bmeg}$ (Figure 3.12 (A)). Consequently, some extracellular component must be produced or liberated by a population of *B. megaterium* overproducing $ComK_{Bmeg}$. This component is not sufficient for effective cell lysis either because its concentration within the growth medium was too low or because it needs an additional cell-associated component of $ComK_{Bmeg}$ overproducing cells for complete lysis of individual cells.

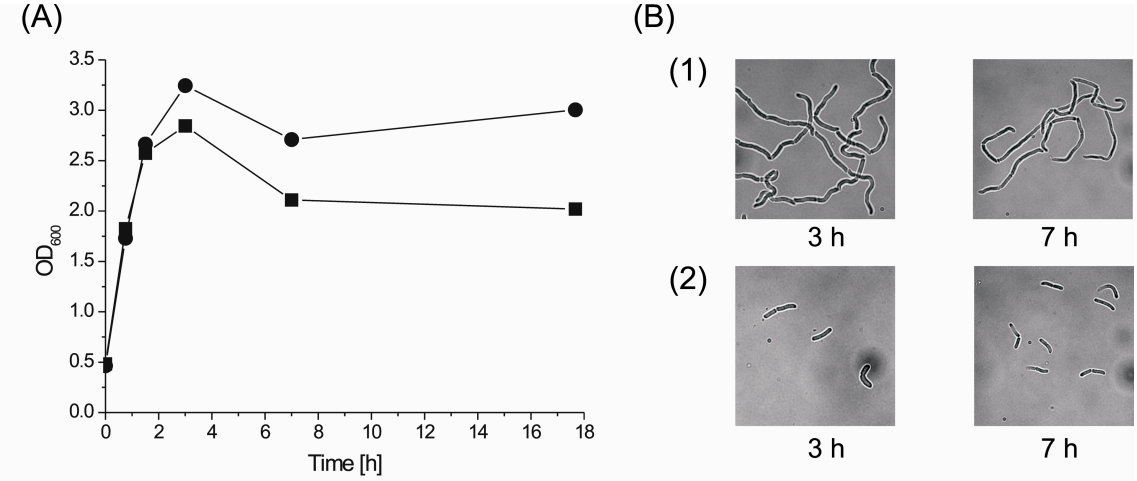

Figure 3.13: Evaluation of extracellular lysin production upon ComK$_{Bmeg}$ overproduction. The *B. megaterium* plasmid strain pDFcomK$_{Bmeg}$ was cultivated in LB medium at 37°C. Recombinant *comK*$_{Bmeg}$ expression was induced by the addition of 1 % (w/v) xylose. *B. megaterium* bearing the plasmid pMM1520 was used as reference and treated in the same manner. After a period of 7 h the cells of each culture were pelleted by centrifugation. The supernatants were sterile filtered and added seperately to a final volume of 50 % (v/v) to an exponentially growing culture of *B. megaterium* in LB medium. The resulting cultures were incubated at 37°C. **(A)** The OD_{600} was determined over a period of 18 h for the culture which was supplemented with the cell-free medium of the reference culture (■) and for the one with the medium of the ComK$_{Bmeg}$ overproducing culture (●). **(B)** After a period of 3 h and 7 h microscopic pictures were taken with 1,000x magnification. **(1)** *B. megaterium* grown in culture medium derived from *B. megaterium* pMM1522 as well as from **(2)** *B. megaterium* pDFcomK$_{Bmeg}$ was analysed with respect to morphological changes.

The data of remarkable changes on the cell morphology and the growth behaviour of *B. megaterium* overproducing ComK$_{Bmeg}$ show striking similarities to the competence associated phenomena of fratricide in *S. pneumoniae* and of autolysis in *S. mutans*, respectively. Consequently, the presented results indicate that the putative transcription factor ComK$_{Bmeg}$ might indeed have a central role in the development of natural competence in *B. megaterium*. However, the influence of ComK$_{Bmeg}$ overproduction on cell lysis differs significantly from the situation in *B. subtilis* 168. Similar results in which its central transcription factor for the development of natural competence triggers changes in cell morphology and cell lysis has not been described so far. Thus, a possible link between natural competence and cell lysis may represent a completely new feature for a species of the genus *Bacillus* which ensures fast spreading of new genetic traits within a population of naturally competent *B. megaterium*.

3.3.6 Influence of ComK on the Transcriptional Activation of Late Competence Genes

To further investigate whether $ComK_{Bmeg}$ can activate the transcription of late competence genes in *B. megaterium* and thereby lead to the formation of a functional DNA-uptake machinery, promoter-GFP fusion assays were performed. For this purpose the previously outlined plasmid strains containing $pDFcomK_{Bmeg}$ in addition to either pDFrecA-gfp or pDFcomG-gfp were employed (chapter 3.3.4). The respective promoter regions of *B. subtilis* 168 contain a K-box and are known to be activated via ComK. The presence of a K-box could also be computationally predicted within the putative promoter region of *recA1* from *B. megaterium*. However, this does not account for the promoter region of the predicted *comG*-operon (Table 3.4).

If $ComK_{Bmeg}$ indeed represents the central transcription factor for the development of natural competence in *B. megaterium*, its overproduction should lead to the transcriptional activation of the assumed promoters of *recA1* and of the *comG*-operon, respectively. This activation should result in the formation of detectable levels of GFP. To further elucidate if ComK from *B. subtilis* 168 (here reffered to $ComK_{Bsu}$) can also serve as positive regulator for the transcription of late competence genes in *B. megaterium* an additional expression plasmid was constructed. Similar to the design of $pDFcomK_{Bmeg}$ the gene encoding $comK_{Bsu}$ was cloned under control of the xylose-inducible promoter into plasmid pMM1622. *B. megaterium* was transformed with the resulting plasmid $pDFcomK_{Bsu}$. In a second step this plasmid strain was finally transformed with either pDFrecA-gfp or pDFcomG-gfp.

Expression analyses were performed in minimal medium in 96-well plates. Expression of $comK_{Bmeg}$ and $comK_{Bsu}$ was induced with 2 % (w/v) xylose, respectively. The OD_{600} as well as the relative fluorescence of GFP was measured every hour. Additionally, samples were taken and analysed via fluorescence microscopy.

In analogy to the results of cultivations in shaking flasks (Figure 3.11) $ComK_{Bmeg}$ overproduction led to a significant breakdown in cell densities in the 96-well plate format. This was not the case for the recombinant production of $ComK_{Bsu}$ (data not shown). Therefore, normalisation of the fluorescence intensity to the OD_{600} of each culture appeared necessary in order to get comparable results. The normalised relative fluorescence gradually increased with time after induction of $comK_{Bmeg}$ expression (Figure 3.14). This observation points to a direct or indirect influence of $ComK_{Bmeg}$ on the transcriptional activation of the assumed promoter regions of the putative late competence gene *recA1* and of the *comG*-operon.

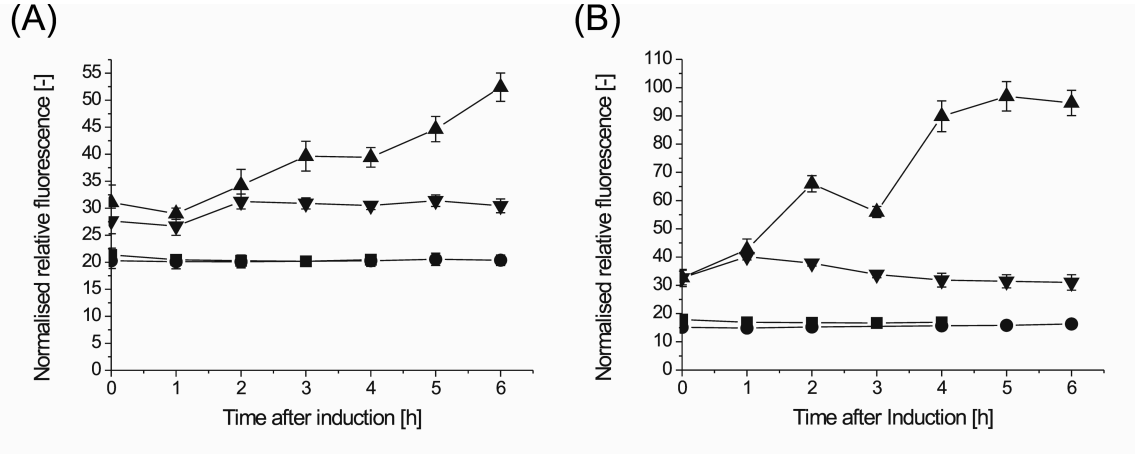

Figure 3.14: Promoter GFP fusion assays. The influence of $ComK_{Bmeg}$ and $ComK_{Bsu}$ on the transcriptional activation of the putative promoter regions of the late competence genes **(A)** *recA1* and **(B)** *comG* were tested with promoter GFP fusion assays. Respective *B. megaterium* plasmid strains were cultivated in minimal medium at 37°C in 96-well plates. If not otherwise indicated 2 % (w/v) xylose were added approximately 3.5 h after the beginning of the cultivation. The OD_{600} as well as the relative fluorescence of GFP was measured every hour and the relative fluorescence was normalised to the cell density of each respective culture. The following strains are represented by the symbols within the diagrams: **(A)** *B. megaterium* with pDFcomK$_{Bmeg}$ and pDFrecA-GFP (▲), *B. megaterium* with pDFcomK$_{Bmeg}$ and pDFrecA-GFP without the addition of xylose (▼), *B. megaterium* with pStop1622 and pDFrecA-GFP (●) and *B. megaterium* with pDFcomK$_{Bsu}$ and pDFrecA-GFP (■). **(B)** *B. megaterium* with pDFcomK$_{Bmeg}$ and pDFcomG-GFP (▲), *B. megaterium* with pDFcomK$_{Bmeg}$ and pDFcomG-GFP without the addition of xylose (▼), *B. megaterium* with pStop1622 and pDFcomG-GFP (●) and *B. megaterium* with pDFcomK$_{Bsu}$ and pDFcomG-GFP (■).

Interpreting these data one has to consider that the observed rise in relative fluorescence might also be an artefact due to the cell lysis upon *comK*$_{Bmeg}$ expression. Indeed, basal transcription occurred from both putative promoter regions even without $ComK_{Bmeg}$ overproduction and no clear increase in the number of GFP producing cells after induction of recombinant *comK*$_{Bmeg}$ expression could be monitored via fluorescence microscopy (Figure 3.15). Instead, the fluorescence within the cell-free culture supernatant increased due to the liberation of GFP from lysed *B. megaterium* cells (data not shown). Consequently, the presence of intra- as well as extracellularly located GFP within cultures overproducing $ComK_{Bmeg}$ impedes statements about the actual increase in GFP formation upon $ComK_{Bmeg}$ production compared to the reference cultures without recombinant expression of *comK*$_{Bmeg}$. Cell lysis and the subsequent liberation of GFP might therefore overestimate the actual influence of $ComK_{Bmeg}$ on the activation of the putative promoter regions of *recA1* and *comG*.

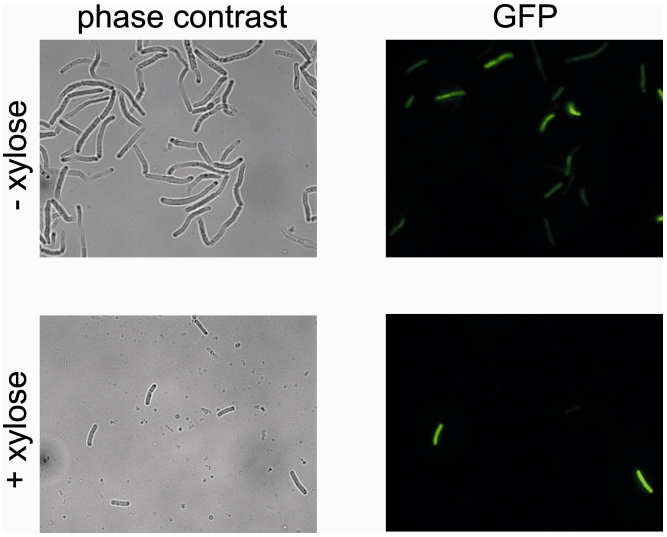

Figure 3.15: Reporter GFP production at single cell level. The *B. megaterium* plasmid strain pDFcomK$_{Bmeg}$/pDFcomG-GFP was cultivated in minimal medium at 37°C. One culture was supplemented with 2 % (w/v) xylose to induce recombinant ComK$_{Bmeg}$ overproduction whereas the second culture was left untreated. 23 h after the induction of *comK*$_{Bmeg}$ expression microscopic images were taken with 1,000x magnification at phase contrast and at fluorescence excitation.

Although ComK$_{Bmeg}$ exhibits homology to ComK$_{Bsu}$ both proteins seem to have a different mode of action in *B. megaterium*. Overproduction of ComK$_{Bsu}$ did not result in dramatic changes in the morphology and the growth behaviour of *B. megaterium*. Moreover, it did not result in the transcriptional activation of the tested putative promoter regions although at least the promoter region of *recA1* exhibits a computationally predicted K-box. In *B. subtilis* 168 it has been shown that a K-box is sufficient for binding of ComK$_{Bsu}$ (Hamoen *et al.* 1998). However, in addition further yet unknown promoter upstream elements are necessary for the stabilisation of RNA-polymerase binding and thus for its transcriptional activation (Susanna *et al.* 2004). Possibly, the putative promoter region of *recA* from *B. megaterium* lacks such upstream-motifs and consequently RNA polymerase binding cannot be initiated by ComK$_{Bsu}$.

3.3.7 Transformation of *Bacillus megaterium* by Means of Natural Competence

The presented results indicated that computationally predicted ComK$_{Bmeg}$ might indeed represent a transcription factor with a link to the development of natural competence in *B. megaterium*. Thus, as a proof of concept the ability of *B. megaterium* to actively take up exogenous DNA was studied. For this purpose the *B. megaterium* plasmid strain

pDFcomK$_{Bmeg}$ was cultivated in parallel in minimal medium suited for the development of natural competence (Mironczuk *et al.* 2008). After reaching an OD_{600} of 0.75 one culture was supplemented with 2 % (w/v) xylose in order to induce recombinant expression of *comK*$_{Bmeg}$ whereas the second culture was left untreated. After 3.5 h of further cultivation samples were taken and incubated with varying concentrations of plasmid pSKE194 (Nahrstedt *et al.* 2005) and with genomic DNA of the *B. megaterium* strain YYBm1 (Yang *et al.* 2006), respectively. The plasmid as well as the genomic DNA harbours a gene which confers resistance to the antibiotic erythromycin. Consequently, the samples were plated onto agar plates containing 2 µg ml^{-1} erythromycin and incubated at 30°C. Colonies arising under these selective conditions were further plated onto a second erythromycin containing-agar plate and checked microscopically for the identity of *B. megaterium*.

Individual colonies were further analysed via PCR for the presence of the gene which confers resistance to erythromycin. As can be seen in Figure 3.16 the presence of the corresponding gene could be verified in the *B. megaterium* plasmid strain pDFcomK$_{Bmeg}$ which overproduced ComK$_{Bmeg}$ and was transformed with the plasmid pSKE194. Interestingly, *B. megaterium* without recombinant overexpression of *comK*$_{Bmeg}$ could also be successfully transformed with genomic DNA of the *B. megaterium* strain YYBm1.

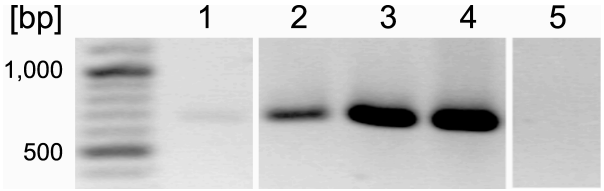

Figure 3.16: Detection of the erythromycin resistance gene via PCR. The cellular DNA of individual clones of the *B. megaterium* plasmid strain pDFcomK$_{Bmeg}$ transformed with either the plasmid pSKE194 or with genomic DNA of *B. megaterium* YYBm1 was prepared. This DNA was subsequently used as template in a PCR reaction to amplify the erythromycin resistance gene. The PCR reactions were separated electrophoretically using the GeneRuler^{TM} DNA Ladder Mix (Fermentas, St.Leon-Rot, Germany) as size standard. Total DNA derived from the following strains were used as templates: *B. megaterium* pDFcomK$_{Bmeg}$ without recombinant overproduction of ComK$_{Bmeg}$ transformed with 1 µg of genomic DNA from *B. megaterium* YYBm1 **(lane 1)** and *B. megaterium* pDFcomK$_{Bmeg}$ with recombinant overproduction of ComK$_{Bmeg}$ transformed with 600 ng of plasmid pSKE194 **(lane 2)**. Plasmid pSKE194 **(lane 3)** and genomic DNA of *B. megaterium* YYBm1 **(lane 4)** were used as template in the positive control reaction and chromosomal DNA of *B. megaterium* DSM319 **(lane 5)** as template in the negative control reaction.

These results indicate that under the chosen conditions *B. megaterium* develops a functional machinery for DNA uptake and recombination. Thus, for the first time

B. megaterium has been shown to develop competence for natural genetic transformation. This feature will possibly greatly enhance the genetic accessibility and the speed for genetic manipulations of this industrially important bacterium in the near future.

3.4 *Bacillus megaterium* – an Alternative Host for the Production of Recombinant Proteins of Eukaryotic Origin

In recent years, *B. megaterium* has successfully been employed for the extracellular production of prokaryotic proteins. Results on the production of a sugar modifying enzyme (Biedendieck *et al.* 2007a), a penicillin amidase (Yang *et al.* 2006) and a polyester-cleaving hydrolase (Yang *et al.* 2007) have recently been published. The recombinant production of eukaryotic model proteins in *B. megaterium* on the other hand has not been studied in detail so far.

3.4.1 Case Study I: Production of a Human Keratin-Binding Domain

Keratin-binding domains (KBDs) are scaffolds having high affinities towards keratin filaments and are aimed to be applied as additives in cosmetics for hair or skin. The subdomain B of human desmoplakin (chapter 1.2.1) has been shown to selectively bind to keratin filaments. First trials at the BASF SE (Ludwigshafen, Germany) to recombinantly produce this subdomain (termed KBD B) in *E. coli* turned out to be problematic since almost exclusively improperly folded protein aggregates were formed. Refolding of the KBD B into its native conformation finally succeeded and the selective binding of the recombinant domain to human hair and skin was demonstrated. Moreover, the covalent attachment of various effector molecules to the KBD B was achieved and filed in patents (Barg *et al.* 2007b; Barg *et al.* 2009). However, due to the laborious refolding procedure the production of KBD B in *E. coli* in industrial scale turned out not to be economical (as of 2005).

3.4.1.1 Rational Evaluation of Keratin-Binding Domain Production

In this study, the feasibility of secretory KBD B production in *B. megaterium* was studied. First experiments at the BASF SE (Ludwigshafen, Germany) indicated that in case of expressing the native *kbd b* gene in *B. megaterium* no recombinant protein production could be detected (personal communication with Dr. Heiko Barg, BASF SE,

Germany). Closer genetic analysis indicated that human *kbd b* has a low codon adaptation to *B. megaterium* (data not shown).

In order to improve the production process on the level of translation, the codon usage was adapted to the one of *B. megaterium*. A corresponding codon-optimised gene was chemically synthetised by Geneart AG (Regensburg, Germany). In addition to the coding region of KBD B, the synthetic constructs encode a N-terminal linker peptide and either a N- or C-terminally located His_6-tag. While the linker comprises a free cysteine residue and is of importance for the covalent coupling of effector molecules, the His_6-tag enables purification of recombinantly produced KBD B from the culture supernatant.

Plasmid pMM1525 and its derivatives were used for cloning of the synthetic constructs. These vectors originate from plasmid pMM1522 and allow for the production of secretory proteins. For this purpose they encode the signal peptide of the *B. megaterium* esterase LipA (SP_{lipA}). Both artificial *kbd b* constructs were fused individually to the coding region of SP_{lipA} under transcriptional control of the xylose inducible promoter (P_{xylA}). The resulting plasmids were termed pP_{xylA}-his_6-kbdb and pP_{xylA}-kbdb-his_6, respectively (Figure 3.17).

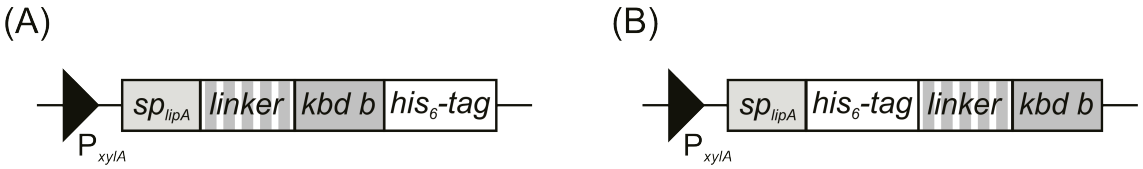

Figure 3.17: Genetic elements of the plasmids (A) pP_{xylA}-kbdb-his_6 and (B) pP_{xylA}-his_6-kbdb. Codon optimised *kbd b*, comprising the coding region for a linker peptide, was fused to sp_{lipA}. Plasmids pP_{xylA}-kbdb-his_6 and pP_{xylA}-his_6-kbdb differ in the localisation of the His_6-tag.

B. megaterium MS941 was used as recombinant production host. Since this strain lacks the major extracellular protease NprM (Wittchen and Meinhardt 1995) it is particularly suited for the production of extracellular proteins (Malten 2005). Comparative expression analyses were performed using the three different cultivation media LB-, TB- and A5+4 medium. TB medium is a standard medium which is rich in nutrients and has already been proven to be suited best for the production of recombinant antibody fragments in *B. megaterium* (Jordan *et al.* 2007). A5+4 medium on the other hand was especially developed for high cell density cultivations of *B. megaterium* in bioreactors (Malten *et al.* 2005a).

As can be seen in western blot analyses (Figure 3.18 (A)), cultivation of the plasmid strain *B. megaterium* MS941 carrying pP_{xylA}-kbdb-his_6 in A5+4 medium resulted in highest yields of KBD B within the culture supernatant. Using this medium, a cultivation period of 9 h was determined to be optimal. Localisation of the His_6-tag (N- or C-

terminal) turned out to have minor effects on KBD B production and secretion (Figure 3.18 (B)). Based on these observations all further studies to elevate KBD B yields were performed in A5+4 medium and aimed to produce KBD B fused to a N-terminal His_6-tag.

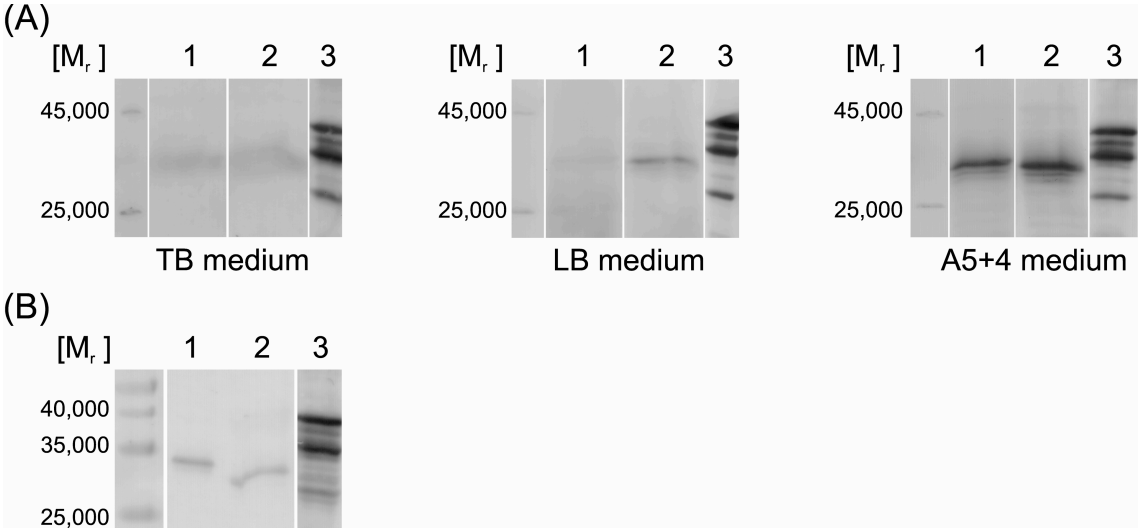

Figure 3.18: Influence of medium composition and His_6-tag localisation on recombinant KBD B production. (**A**) The *B. megaterium* plasmid strain MS941 pP_{xylA}-kbdb-his_6 was cultivated in indicated media at 37°C. Expression of *kbd b* was induced at an OD_{578} of 0.4 by the addition of 0.5 % (w/v) xylose. Samples were taken 6 h (1) and 9 h (2) after induction of recombinant gene expression. Extracellular proteins were precipitated using 44 % (w/v) ammonium sulphate. Proteins equivalent to 1.5 ml of cell-free culture supernatant were separated via 15 % SDS-PAGE and transferred onto a PVDF membrane. Recombinant KBD B was detected immunologically using a KBD B specific primary antibody (BASF SE, Ludwigshafen, Germany). Approximately 1 µg of purified recombinant KBD B from *E. coli* (BASF SE, Ludwigshafen, Germany) was used as positive control (3). (**B**) The *B. megaterium* plasmid strains MS941 carrying pP_{xylA}-kbdb-his_6 (1) or MS941 carrying pP_{xylA}-his_6-kbdb (2) were cultivated as indicated above in A5+4 medium. Samples were taken 9 h after induction of recombinant gene expression and detection of KBD B within the cell-free culture supernatant was performed as described above. 1 µg of purified recombinant KBD B from *E. coli* (BASF SE, Ludwigshafen, Germany) was used as positive control (3).

Besides medium composition, the cultivation temperature is of central importance for the quantity and quality of a given recombinant protein (Jordan *et al.* 2007). However, both cultivation parameters are strongly dependent on the nature of the target protein and have to be determined experimentally. The influence of three different cultivation temperatures on recombinant KBD B production and secretion were analysed. While almost no extracellular KBD B was produced at 30°C and 42°C, significantly higher amounts of KBD B were detected in the culture supernatant at 37°C (Figure 3.19 (A)).
Finally, the choice of an adequate *B. megaterium* production strain influences the yield in recombinant protein production. The *B. megaterium* strain WH323 *e.g.* has been

proven to be better suited for the secretory production of a heterologous hydrolase than *B. megaterium* MS941 (Yang *et al.* 2007). Therefore, this strain was also chosen as alternative host for the production and secretion of recombinant KBD B. Cultivation of the corresponding *B. megaterium* plasmid strain WH323 carrying pP_{xylA}-his_6-kbdb in A5+4 medium at a cultivation temperature of 37°C resulted in higher yields of KBD B within the culture supernatant than by using the *B. megaterium* strain MS941 (Figure 3.19 (B)).

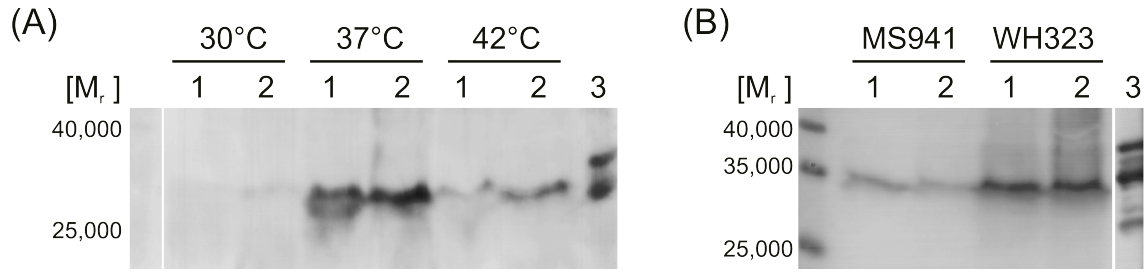

Figure 3.19: Influence of cultivation temperature and *B. megaterium* host strain on recombinant KBD B production. (**A**) The *B. megaterium* plasmid strain MS941 pP_{xylA}-his_6-kbdb was cultivated in A5+4 medium at indicated temperatures. Expression of *kbd b* was induced at an OD_{578} of 0.4 by the addition of 0.5 % (w/v) xylose. Samples were taken 6 h (1) and 9 h (2) after induction of recombinant gene expression. Extracellular proteins were precipitated using 44 % (w/v) ammonium sulphate. Proteins equivalent to 1.5 ml of cell-free culture supernatant were separated via 15 % SDS-PAGE and transferred onto a PVDF membrane. Recombinant KBD B was detected immunologically using a KBD B specific primary antibody (BASF SE, Ludwigshafen, Germany). 1 µg of purified recombinant KBD B from *E. coli* (BASF SE, Ludwigshafen, Germany) was used as positive control (3). (**B**) The *B. megaterium* plasmid strains MS941 pP_{xylA}-his_6-kbdb and WH323 pP_{xylA}-his_6-kbdb were cultivated in A5+4 medium at 37°C. Expression of *kbd b* was induced at an OD_{578} of 0.4 by the addition of 0.5 % (w/v) xylose. Samples were taken 6 h (1) and 9 h (2) after induction of recombinant gene expression. Detection of KBD B within the cell-free culture supernatant was performed as described above. 1 µg of purified recombinant KBD B from *E. coli* (BASF SE, Ludwigshafen, Germany) was used as positive control (3).

In summary, the adaptation of human *kbd b* codon usage to *B. megaterium* in combination with the identification of an adequate production strain and appropriate cultivation parameters resulted in detectable levels of KBD B within the secretome. However, despite all these optimisation strategies KBD B concentrations were still too low to be visualised by Coomassie Brilliant Blue protein staining and purification of KBD B via its His_6-tag from the culture supernatant was not successful (data not shown).

3.4.1.2 Identification of Bottlenecks in Keratin-Binding Domain Production

When the project on secretory KBD B production started in 2005 there was no indication that KBD B might be harmful to *B. megaterium*. However, despite numerous optimisation strategies concerning the codon usage and cultivation parameters, only low KBD B yields were achieved (chapter 3.4.1.1). Thus, there must be significant bottlenecks limiting secretory KBD B production in *B. megaterium*.

In a population of host cells producing a deleterious protein, cells which cease recombinant gene expression will overgrow the others due to the relief in metabolic burden. Such block in recombinant protein formation may be achieved by selecting for mutations which reduce promoter activity and thereby limit the transcription of the corresponding gene. The subsequent breakdown in mRNA formation can be monitored via northern blot and serves as a good indicator that the harmfulness of the gene product limits its formation.

Northern blot analyses were performed to elucidate whether a decline in *kbd b* expression and a subsequent breakdown in mRNA formation is responsible for low KBD B yields. For this purpose, total RNA fractions of the *B. megaterium* plasmid strain MS941 carrying pP_{xylA}-his_6-kbdb were isolated at different times before and after induction of recombinant *kbd b* expression. Detection of corresponding transcripts was performed using a *kbd b*-specific RNA probe.

Before the induction of recombinant gene expression with xylose no *kbd b* transcripts were detected. This observation is in agreement with former studies demonstrating the stringent control of recombinant gene expression using the xylose-inducible promoter (Biedendieck *et al.* 2007c). After induction of *kbd b* expression high levels of corresponding mRNAs were detected. The varying length of *kbd b* transcripts point to a possible degradation and random termination of transcription since the *kbd b* gene lacks a transcription terminator. The constant high level of mRNA over time does not support the hypothesis that KBD B might be toxic to *B. megaterium* and therefore implicates that transcription does not represent the limiting bottleneck in KBD B production.

Besides the toxicity of a gene product, posttranslational bottlenecks are known to limit protein production and secretion. Multiple factors are reported to influence the efficiency of recombinant protein secretion (chapter 1.4). Proteins being translocated by the Sec-dependent pathway have to adopt a translocation-competent state (*e.g.* an unfolded state) with the help of molecular chaperones (van Wely *et al.* 2001). Thus, protein conformation is of importance for efficient translocation. Also, heterologous

proteins which tend to form insoluble aggregates have been proven to be inefficiently secreted (Bendig 2007; Wu *et al.* 1998).

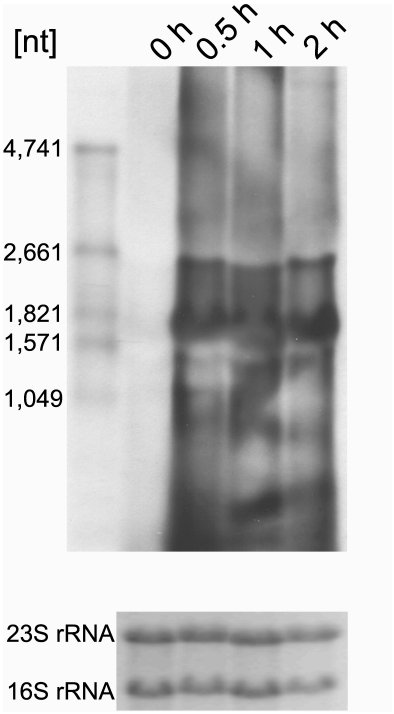

Figure 3.20: Time-dependent transcription of *kbd b* analysed by Northern blotting. The *B. megaterium* MS941 plasmid strain pP_{xylA}-his_6-kbdb was cultivated in A5+4 medium at 37°C. Expression of *kbd b* was induced at an OD_{578} of 0.4 by the addition of 0.5 % (w/v) xylose. Samples were taken before and at indicated times after induction of recombinant gene expression. Total RNA fractions were isolated and separated via agarose gel electrophoresis. Hybridisation of a digoxigenin labelled *kbd b*-specific RNA probe was detected via chemiluminescence using an anti-digoxigenin antibody. The loading control was stained with ethidium bromide and verifies that equal amounts of RNA were applied per lane.

Interestingly, different aggregates of KBD B were identified within the soluble and insoluble intracellular protein fractions of the *B. megaterium* plasmid strain WH323 carrying pP_{xylA}-his_6-kbdb (Figure 3.21). Besides monomers, dimers of KBD B seem to be present in both protein fractions 6 h and 9 h after induction of *kbd b* expression. Additionally, the apparent formation of highly insoluble KBD B tetramers, as well as the presence of multiple KBD B degradation products was detected. These observations underscore that quantitative secretion of KBD B with *B. megaterium* is inhibited by its efficient cytosolic degradation and the formation of KBD B multimers being incompetent for Sec-dependent translocation. Thus, the characteristic of KBD B to readily from multimers in addition to its quick proteolytic degradation within the cytosole reduces secretory KBD B production in *B. megaterium*.

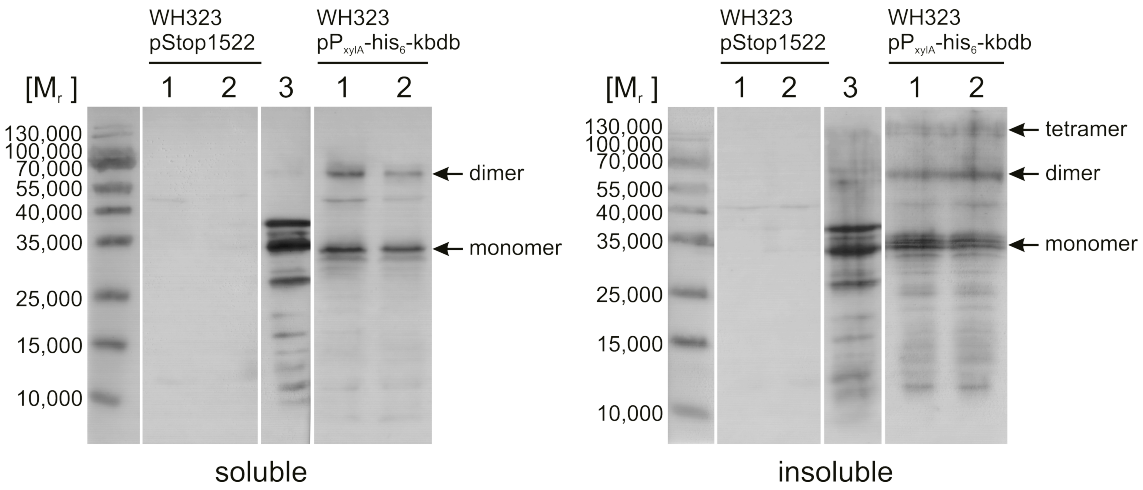

Figure 3.21: Detection of cytosolic KBD B aggregation and degradation. The *B. megaterium* plasmid strains WH323 carrying pP$_{xylA}$-his$_6$-kbdb and WH323 carrying pStop1522 (negative control) were cultivated in A5+4 medium at 37°C. Recombinant gene expression was induced at an OD_{578} of 0.4 by the addition of 0.5 % (w/v) xylose. Samples were taken 6 h (1) and 9 h (2) after induction of recombinant gene expression. Soluble and insoluble intracellular protein fractions were prepared. Proteins equivalent to approximately 5 x 10^8 cells were loaded per lane and subsequently separated via 15 % SDS-PAGE. Proteins were transferred onto a PVDF membrane and recombinant KBD B was detected immunologically using a KBD B specific primary antibody (BASF SE, Ludwigshafen, Germany). 1 µg of purified recombinant KBD B from *E. coli* (BASF SE, Ludwigshafen, Germany) was used as positive control (3). Protein bands corresponding to putative KBD B monomers, dimers and tetramers are indicated.

3.4.2 Case Study II: Production of a Fungal Hydrophobin

Hydrophobins have unique and remarkable properties. Their amphiphilic nature and self-assembling properties are of great interst for a wide range of industrial applications (chapter 1.2.2). However, a broad utilisation of hydrophobins is limited due to difficulties associated with their economical production in large scale. Since hydrophobins consist of four structurally essential disulfide bridges, the cytoplasmatic recombinant production of hydrophobins is problematic. Production trials at the BASF SE with *E. coli* as production host resulted in the formation of insoluble hydrophobin aggregates (as of 2006). Furthermore, the recombinant production of hydrophobins in fungal hosts like *Aspergillus nidulans* resulted in low yields hampering a commercialisation at reasonable prices (as of 2006).

3.4.2.1 Rational Evaluation of Hydrophobin Production

The hydrophobin DewA from *A. nidulans* was used as model to evaluate secretory hydrophobin production in *B. megaterium*. Analysis of the corresponding gene showed

that its codon usage is badly adapted to *B. megaterium* (data not shown). Thus, in a first step the codon usage of *dewA* was computationally optimised for a subsequent recombinant expression in *B. megaterium*. The artificial gene was chemically synthesised by Geneart AG (Regensburg, Germany) including the coding region for its natural signal peptide in addition to the coding region for a C-terminally located His_6-tag. The synthetic construct was cloned into the plasmid pStop1622 under transcriptional control of the xylose-inducible promoter. The *B. megaterium* strain MS941 was transformed with the resulting plasmid pP_{xylA}-sp_{native}-dewA-his_6. Expression analyses were performed in LB medium at 37°C.

The native unprocessed DewA comprises a signal peptide which targets this hydrophobin to the surface of *A. nidulans* cells. Although the process of protein translocation is highly divergent between eukaryotes and prokaryotes, the signal peptide of DewA was computationally predicted by neural networks as well as hidden Markow models to be recognised by Gram-positive bacteria (Bendtsen *et al.* 2004). However, no recombinant DewA production could be detected within the culture supernatant of the *B. megaterium* plasmid strain MS941 carrying pP_{xylA}-sp_{native}-dewA-his_6 (data not shown).

In the next step, a signal peptide of Gram-positive origin was used for the translocation of DewA. Therefore, the codon-optimised *dewA* construct without the coding region for the native signal peptide was cloned into the secretion vector pMM1525 (chapter 3.4.1.1). Thereby, it was fused to the coding region of the homologous signal peptide SP_{lipA} under transcriptional control of the xylose inducible promoter. The *B. megaterium* strain MS941 was transformed with the resulting plasmid pP_{xylA}-sp_{lipA}-dewA-his_6.

Expression analyses in LB medium at 37°C showed that DewA was successfully produced and secreted after changing the heterologous, native signal peptide into a homologous one. Besides LB medium, TB- and A5+4 medium were tested for their influence on the recombinant production of DewA. Interestingly, out of these three cultivation media solely LB medium was suited for recombinant DewA production (Figure 3.22). Highest yields of DewA within the culture supernatant were obtained 6 h after induction of recombinant gene expression. Extended cultivation times resulted in diminished DewA yields. Thus, *B. megaterium* seems to form elevated amounts of extracellular proteases in the transition from the exponential to the stationary growth phase which degrade recombinant DewA.

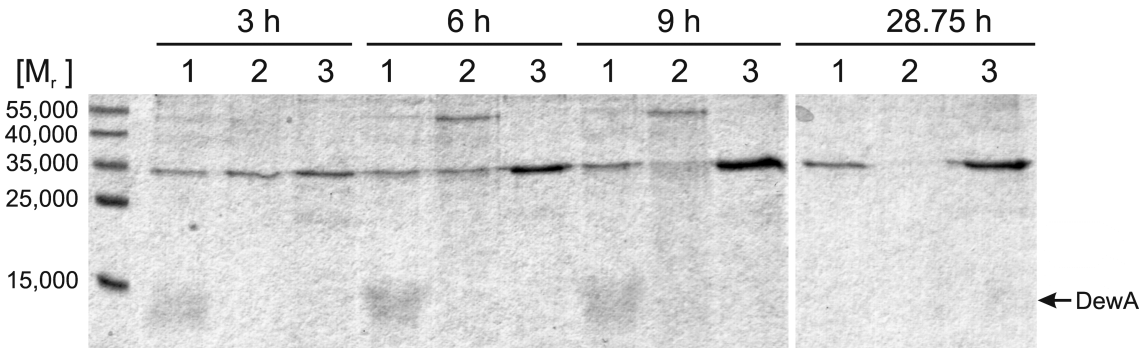

Figure 3.22: Influence of medium composition on recombinant DewA production. The *B. megaterium* plasmid strain MS941 pP_{xylA}-sp_{lipA}-dewA-his_6 was cultivated at 37°C in LB- (1), A4+4-(2) and TB-medium (3), respectively. Expression of *dewA* was induced at an OD_{578} of 0.4 by the addition of 0.5 % (w/v) xylose. Samples were taken at indicated times after induction of recombinant gene expression. Extracellular proteins were precipitated using 15 % (w/v) ammonium sulphate. Proteins equivalent to 1.5 ml of cell-free culture supernatant were separated via 15 % SDS-PAGE and stained with Coomassie Brilliant Blue.

The *B. megaterium* strain DSM319 encodes several minor extracellular proteases in addition to the major extracellular protease NprM. Homologous to the serine protease Vpr and the aminopeptidase YwaD from *B. subtilis* 168 are found within the culture supernatant of *B. megaterium* MS941 (Wang *et al.* 2006a). Additionally, during high cell density cultivations a stress-related zinc-dependent metalloprotease was detected within the secretome of *B. megaterium* MS941 (Wang *et al.* 2006b).

Since the activity of metalloproteases is dependent on divalent cations they can be efficiently inhibited by the chelating agent EDTA. However, the addition of EDTA to cell-free culture supernatants at different times after induction of recombinant gene expression elevated the stability of DewA only in a negligible manner compared to the reference without EDTA treatment. Instead, by lowering the storage temperature from 25°C to -20°C the long-term integrity of DewA could be elevated significantly (Figure 3.23).

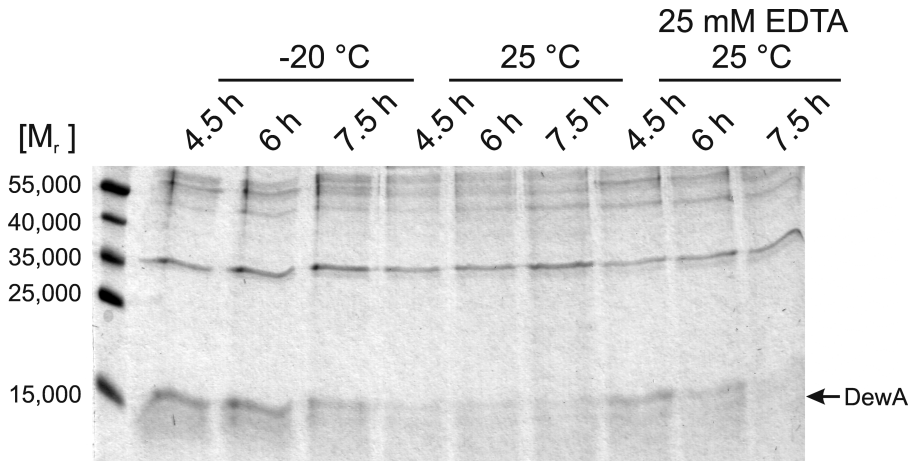

Figure 3.23: Stability of recombinant DewA within culture supernatants. The *B. megaterium* plasmid strain MS941 pP_{xylA}-sp_{lipA}-dewA-his_6 was cultivated at 37°C in LB medium. Expression of *dewA* was induced at an OD_{578} of 0.4 by the addition of 0.5 % (w/v) xylose. Samples were taken at indicated times after induction of recombinant gene expression. Cell-free culture supernatants were stored at indicated temperatures with or without the addition of EDTA for approximately 17 h. Extracellular proteins were precipitated using 15 % (w/v) ammonium sufate. Proteins equivalent to 1.5 ml of cell-free culture supernatant were separated via 15 % SDS-PAGE and stained with Coomassie Brilliant Blue.

3.4.2.2 Properties of Purified Hydrophobin

Recombinant DewA derived from the *B. megaterium* plasmid strain MS941 pP_{xylA}-sp_{lipA}-dewA-his_6 could be purified to homogeneity via the C-terminally located His_6-tag by a simple and convenient one step purification procedure. By applying Ni-NTA Sepharose directly to the cell-free culture supernatant, extracellular DewA without any visible impurities as determined via SDS-PAGE could be obtained (Figure 3.24).

To further test whether recombinant DewA from *B. megaterium* is properly folded and consequently exhibits surface active properties, additional studies were performed at the BASF SE (Ludwigshafen, Germany). The surface modifying characteristics of hydrophobins were evaluated by coating hydrophilic and hydrophobic surfaces with purified recombinant DewA from *B. megaterium*, respectively. Experiments were performed with glass as representative for hydrophilic surfaces and Teflon® as representative for hydrophobic surfaces. Changes in the contact angle of a water droplet on coated and uncoated surfaces was considered as a measure for the surface active properties of DewA.

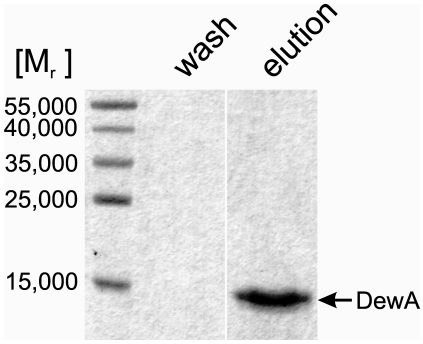

Figure 3.24: Purification of recombinant DewA from the culture supernatant. The *B. megaterium* plasmid strain MS941 pP_{xylA}-sp_{lipA}-dewA-his_6 was cultivated at 37°C in LB medium. Expression of *dewA* was induced at an OD_{578} of 0.4 by the addition of 0.5 % (w/v) xylose. 4.5 h after induction of recombinant gene expression the bacteria were pelleted by centrifugation. Ni-NTA sepharose was added to the cell-free culture supernatant. The suspension was incubated for 30 min at room temperature. Afterwards, Ni-NTA Sepharose was pelleted by centrifugation and washed twice with 1.5 column volumes of 50 mM Tris-HCl (pH 8.0). Elution of recombinant DewA was performed with 200 mM imidazole in 50 mM Tris-HCl (pH 8.0). Proteins equivalent to 1.2 ml of cell-free culture supernatant were separated via 15 % SDS-PAGE and stained with Coomassie Brilliant Blue.

As can be seen in Table 3.5 the contact angle of a water droplet on glass is 22.9° higher for the DewA-coated surface compared to the unmodified surface. Clearly, the DewA-coating decreased the polarity of the glass surface. Similar to that, coating of DewA onto Teflon® massively increased the surface polarity. Thus, DewA produced with *B. megaterium* is functional and exhibits highly surface modifying properties.

Table 3.5: Coating of surfaces with recombinant DewA from *Bacillus megaterium*. Glass and Teflon® surfaces were incubated over night at 80°C in coating buffer (50 mM Tris-HCl (pH 8.0), 1 mM $CaCl_2$) containing 50 µg ml^{-1} of purified recombinant DewA. After incubation, the surfaces were washed with water, incubated for 10 min at 80°C in 1 % (w/v) SDS and washed with water again. The surfaces were dryed at room temperature and the contact angle of a water droplet with a volume of 5 µl on DewA-coated surfaces was determined. Contact angles of water droplets on uncoated surfaces were taken as control.

	Glass Contact Angle [°]	**Teflon® Contact Angle [°]**
Uncoated	11.2	110.3
Coated	34.1	40.6

Summarised, as proof of concept a structurally complex eukaryotic protein of industrial importance with a high content of intramolecular disulfide bridges could be functionally produced and secreted with *B. megaterium*. The surface-active properties of purified DewA from *B. megaterium* exhibit even better characteristics than those of DewA recombinantly produced in *E. coli* (personal communication with Dr. Thomas Subkowski, BASF SE, Ludwigshafen, Germany). However, the structural instability of

DewA within the culture supernatant emphasises the need for further genetic optimisations of *B. megaterium* with respect to targeted deletions of proteases limiting the yield of a given recombinant protein.

4 SUMMARY

The work presented in this thesis focused on the directed genetic optimisation of the Gram-positive bacterium *B. megaterium* for recombinant protein production. For this purpose gene expression was systematically analysed and improved on transcriptional and translational level.

A novel gene expression system was developed based on the highly processive RNA polymerase of the bacteriophage T7. Using this system, GFP was successfully produced intracellularly to significant higher amounts (49.7 mg l^{-1}) compared to the reference using the well-established xylose-inducible promoter system (7.2 mg l^{-1}). Interestingly, the application of the T7 RNA polymerase dependent gene expression system for the secretory production of the exoenzyme levansucrase resulted in significantly lower protein yields possibly due to an overload of the secretion machinery and the subsequent induction of stress responses.

Furthermore, a versatile codon test system was developed and individual codons were experimentally identified which are limiting the efficiency of the translational process in *B. megaterium*. The subsequent identification of a rare tRNA gene and its coexpression significantly elevated the rate of translation of corresponding codons into the respective polypeptide chain as estimated by the formation of the model protein GFP.

First insights into the molecular basis for the development of natural competence in *B. megaterium* were obtained. Overproduction of the putative central transcription factor ComK for competence development results in partial cell lysis. Nevertheless, for the first time the ability of *B. megaterium* to develop a functional machinery for DNA-uptake and -integration was demonstrated.

As a proof of concept two eukaryotic proteins of industrial importance, a human keratin-binding domain and a fungal hydrophobin, were produced with *B. megaterium* in cooperation with the BASF SE. Multiple parameters like codon usage optimisation, the nature of the signal peptide or the cultivation conditions were analysed in order to improve the yield in secretory production of the respective proteins. It was shown that intracellular aggregation and degradation severely hampered quantitative production of the keratin-binding domain. The fungal hydrophobin on the other hand could successfully be produced, secreted and purified to homogeneity and its surface active properties were demonstrated.

Overall, the results of this thesis provide a solid basis for broadening the genetic amenability of the industrially important *B. megaterium* in the near future.

5 OUTLOOK

The economic production of recombinant proteins for industrial applications is of central importance for building up smart technologies which are increasingly independent of traditional oil-based production processes. The studies presented in this thesis point to different optimisation strategies which may lead to an increased yield in recombinant protein production by using *B. megaterium* as protein production host:

1) The influence of the coexpression of additional rare tRNA genes on the efficiency of the translational process should be studied in more detail. In the end, this may lead to the development of a *B. megaterium* strain which coexpresses all rare tRNA genes. This strain would be valuable for the production of heterologous proteins whose formation would otherwise be limited due to a bad codon adaptation to *B. megaterium*.
2) Further insights into the secretion stress response of *B. megaterium* during high-level production of secretory proteins are necessary. Whole transcriptome analyses may lead to the identification of genes whose activation or repression correlates with diminished yields in secretory protein formation. These genes may either represent markers useful for the fine-tuning of recombinant gene expression or are targets for gene-knockouts or -knockins and the rational generation of optimised protein production strains.
3) In order to realise a simplified method for the generation of directed genomic mutagenesis the whole process of natural competence development should be further evaluated. From a fundamental point of view the molecular basis underlying the competence associated phenomenon of cell lysis should be analysed with the help of transcriptome analyses. Moreover, it would be interesting to reveal whether overproduction of the central transcription factor for competence development leads to autolysis or to the lysis of siblings. Latter represents a related mechanism to competence-induced fratricide of the human pathogen *S. pneumoniae*. Finally, an optimised method for the generation of naturally competent *B. megaterium* has to be established and first directed gene-knockouts by means of natural competence have to be performed.

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